NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR
Annual Technical Report
1996-1997

19980921 070

Submitted by

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Period Covered: 20 September, 1996 through 19 September, 1997

Cooperative Agreement DAMD17-93-V-3013

between

United States Army Medical Research and Development Command
(Walter Reed Army Institute of Research)

and

Louisiana State University Medical Center
Neuroscience Center of Excellence

Volume 6 of 9

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Protecting the Auditory System and Prevention of Hearing Problems

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5. **ABSTRACT: (SPECIFIC AIMS)** (Maximum of 200 words)

ANIMAL PROJECT: The **SPECIFIC AIMS** of this study are to demonstrate and explore mechanisms for preventing the effects of intense sound. In years 01, 02, 03 we discovered that continuous, ipsilateral sound stimulation (CM-LIPS) will produce complex changes in the mechanics of the cochlea. In year 04 we obtained additional evidence that ATP is involved in generating this mechanics. We completed the noise exposure studies and found that continuous noise is less effective than interrupted noise in inducing "toughening". Cellular mechanism studies discovered that ATP is not only a cytotoxin but also a mitogen, indicating that ATP may be a key player in noise-induced deafness, "toughening" and cell growth.

HUMAN PROJECT: We have found that binaural noise suppresses linear click evoked emissions twice as much as ipsilateral noise and 3 times as much as contralateral noise. However, of 39 subjects enrolled and 30 completely tested so far in the multi-day protocol, subjects with noise exposure effects at higher frequencies show LESS suppression to clicks in the left ear than do subjects with no hearing loss. This may support the original hypothesis in this program that "Noise Tender Ears" will show different emission suppression patterns from ears that are tough and supports the observation that, in general, males tend to show more left ear noise trauma than right ear noise trauma.

6. **INTRODUCTION (Hypothesis)**: The following text is approximately the same as in the Progress Reports for years # 01- # 03. The nature of the problem that this proposal addresses is that soldiers may be exposed to intense noise hazards which will affect their hearing. The hypothesis to be tested is that noise induced hearing loss can be prevented or attenuated.

The **animal experimental literature** demonstrates that chronic low level sound will "toughen" ears so subsequent intense noise will induce less damage (e.g., Canlon et al., 1988; Campo et al., 1991; Franklin et al., 1991). One hypothesis regarding this phenomena is that low level sound activates inhibitory cholinergic efferents that reduce the effects of intense sound by attenuating cochlear partition motion. Alternatively, there may be down-regulation of a protein that has a role in noise induced hearing loss. ATP may be released together with ACh from the efferents. In years #02 - #04 we obtained data indicating that the neurotransmitter, ATP, may play a major role in noise effects in the cochlea because we demonstrated that the ATP receptor on OHCs may be down-regulated during noise exposure (Chen et al., 1995). ATP may be a major player in the effects of toughening because it has powerful effects on the OHCs and supporting cells and ATP is known to induce cell death (Bobbin, 1996; Eybalin, 1993; Kujawa et al., 1994b; Valera et al., 1994). If it can be shown that ATP induces cell death in the cochlea then evidence for a role for ATP in noise-induced hearing loss will be obtained.

The **human experimentation program** was proposed to develop emission-based tests to detect abnormal cochlear function rapidly and accurately (Berlin, 1996; Berlin et al., 1995, 1993). The procedure offers the equivalent of a non-invasive acoustic microscope to analyze the integrity of the OHCs; this is an important tool since OHC damage is always seen in humans who have suffered noise damage. The hypothesis is that "noise tender" ears that are particularly

susceptible to noise damage will show different emission suppression patterns from ears that are "tough".

Our intention remains to study 100 musicians, military and/or industrial workers who have had matched exposures to noise in an efferent suppression paradigm. Before starting the final design of this study we laid basic parametric groundwork relative to the nature of the stimuli, their delivery (whether clicks or tones, whether binaural, contralateral only, or ipsilateral), the echo analysis, and whether there are any pertinent gender and ear differences which have to be entered into the final subject selection.

In summary, in **animals** we will test the hypothesis that: (1) the impact of noise on hearing can be lessened; (2) certain classes of drugs may prevent (or exacerbate) noise-induced hearing loss. In **human subjects**, we will explore the concept that some individuals are more or less susceptible to noise damaging effects. We will describe those populations to determine the basis of this "toughness" or susceptibility to damage from noise. We will examine whether the techniques which we may discover aid in preventing noise-induced hearing loss in soldiers.

7. BODY (STUDIES AND RESULTS CONDUCTED UP TO AND DURING THE CURRENT BUDGET YEAR 04):

ANIMAL PROJECT: In years #01 - #04 we have continued to carry out acute and chronic experiments. In brief, the **acute** experiments focus on the effects of ipsilateral sound on the mechanics of the cochlea, and studies of single cells in the whole cell voltage clamp configuration; and the **chronic** experiments examine "toughening" in the intact animal. These experiments follow

directly our stated specific aims for years #01, #02, #03 and #04 and the first three TECHNICAL OBJECTIVES: to extend studies which demonstrate that contralateral, ipsilateral, or "toughening" sound will prevent the effects of intense noise, to test the role of the efferents, and to explore cellular mechanisms.

The methods were described (Kujawa et al., 1992; 1993; 1994a; year #01 progress report; Appendix 2 and 3). Briefly, guinea pigs are anesthetized (urethane: 1.5 gm/kg) and tracheotomized. ECG is monitored and temperature maintained at 38° ± 1°C. The right auditory bulla is exposed, opened and tendons of the middle ear muscles are sectioned. For drug application to the cochlea, holes are placed in the cochlear basal turn: one in scala tympani for the introduction of perfusates and one in scala vestibuli to allow fluid escape. Perfusates are introduced into scala tympani at approximately 2.5 µl/min for 10 min through a pipette coupled to a syringe pump. DPOAEs ($2f_1$-f_2 & f_2-f_1) are recorded in response to primary stimuli (f_2/f_1=1.2) delivered to the right ear of each animal by an acoustic probe/hollow ear bar assembly. Acoustic signals present within the canal are detected by a microphone system (Etymotic Research, ER-10) contained within the probe. Microphone output is directed via a preamplifier (Etymotic Research, ER-1072) to a signal analyzer (Hewlett Packard 3561A). To extract the DPOAE from the canal spectrum, the signal is sampled, digitized and submitted to Fast Fourier Transform (FFT) analysis. The resulting spectrum is averaged (over 25 samples) and displayed at the spectrum analyzer (1 kHz window, 3.75 Hz BW). DPOAE amplitude is defined as the spectral peak corresponding to the DPOAE frequency. The contralateral stimulus is a wideband noise with an overall level of 70 dB SPL and flat from 0.9 to 15.8 kHz.

The **acute** experiment examined the influence of continuous, moderate-level (60 dB SPL) ipsilateral primary stimulation (CM-LIPS) on the f_2-f_1 DPOAE at 1.25 kHz (quadratic). The

stimulation and response monitoring protocols consisted of quiet for 15 min, then turning the primaries on for 9 min, off for 1 min and then on for an additional 3 min. CM-LIPS resulted in a complex change in the magnitude of f_2-f_1 (see Appendix 2, 3, 4, 8 and 21 for publications). In previous years, we ruled out the efferent nerves as contributors to CM-LIPS (year #01, Kujawa et al., 1995; Appendix 2) and described the role of calcium (year #02, Kujawa et al., 1996; Appendix 3). In year #03 and #04 we continued to explore mechanisms for the CM-LIPS-induced alteration in cochlear mechanics. We made a series of major discoveries: both ATP antagonists, suramin and PPADS, blocked the decrease in the quadratic DPOAE during CM-LIPS(Skellett et al., 1997a, Appendix 8; Chen et al., 1997b, Appendix 10; Bobbin et al., 1997a, Appendix 11). Our hypothesis is that ATP is slowly released during CM-LIPS and produces the changes observed in the quadratic DPOAE. This is in harmony with results in year #02 indicating that there is an apparent down-regulation of ATP receptors during "toughening" (Chen et al., 1995; Appendix 1).

In year #04, in the **chronic** experiment, we finished the study described previously which used a "toughening" noise that was more intense (than the 65 dB SPL rms, A-scale used in year #02; Skellett et al., 1996a; Appendix 5) in order to test whether toughening occurs with the intense sound. In addition, we compared the effects of continuous vs. interrupted exposures of equal acoustic energy (experiment #3 in the original proposal). The results show that although both exposures studied had equal acoustic energy, the interrupted noise exposure was significantly less damaging to the cochlea than the continuous noise. In addition, we tested whether these sounds induce toughening as monitored by a decrease in the effects of a subsequent intense sound exposure. The results suggest that overall, there were significant differences in the degree of protection provided by prior exposure to moderate-level continuous vs. interrupted toughening sounds. There

was protection afforded by the interrupted noise protocol, however, the frequency region where protection occurred was limited to the region above that of the noise exposure band. Such results have not been reported previously and may lead to new understanding of the toughening phenomena. There was a lack of protective effect across the entire test frequency range when the continuous noise protocol was used as the moderate-level toughening exposure. The results have been submitted for publication and have been revised according to the reviewers comments (Skellett et al., 1997b; Appendix 17, 18, 20). In the following year we will extend these findings by examining for differences in the ATP receptors of animals treated by this toughening treatment.

To examine the cellular basis for these "toughening"-induced changes in the cochlea (experiment #4 in original application), we carried out whole cell voltage clamp recordings from the OHCs, Deiters' cells, Hensen's cells and pillar cells (Chen et al., 1997b; Appendix 7; Skellett et al., 1997a; Appendix 8). During year 04 we published that rat OHCs do not respond to ATP but that both rat and guinea pig supporting cells do respond to ATP (Chen et al., 1997a; appendix 7, 19). Interestingly, the rat is less sensitive to noise-induced hearing loss, possibly indicating that the rat is already toughened. In addition, we demonstrated that ATP receptors are functionally present on pillar cells and that at the single cell level ATP actions on the ionotropic receptors on cells in the organ of Corti are blocked by suramin and PPADS while cibacron acts on potassium channels (Skellett et al., 1997a; Chen et al., 1997b; appendix 8, 10).

During years #03- #04, we discovered that application of ATP to short term cultured guinea pig OHCs induced a loss of fluid (necrosis) and turned the cells into membrane ghosts. We extended this finding in vivo. Survival of the animal for two weeks after a two hour ATP administration to the cochlea was found to result in death of most of the cells in the cochlea (i.e.,

cytotoxicity) while simultaneously inducing proliferation of fibrocytes (i.e., mitogenicity). The in vivo and in vitro results have been accepted for publication (Bobbin et al., 1997b; Appendix 9, 22). Thus we have formulate the following hypothesis: that intense sound releases excessive ATP and this kills the cells of the cochlea; toughening exposures result in a down regulation of ATP receptors and subsequently less cell death due to intense sound exposure.

HUMAN PROJECT: The goals of the 3 previous years have been completed and 99 subjects have by now been tested in two separate experimental tracks: 60 subjects have been tested in an attempt to develop age and gender norms for efferent emission suppression, and 39 musicians have been tested to date (data on 30 are completed) for tone burst emission suppression patterns. However, these studies all evaluate patients *after* the fact of noise exposure and reveal data that are difficult to interpret unequivocally because of the inherent problems in controlling for levels of noise exposure history in our musicians.

It would be ideal if we could evaluate subjects both before and after known and measured exposure to better predict their susceptibility to noise exposure. Therefore a third experimental track has been initiated in a collaborative effort with Dr. Darrell Wester and his colleagues of the US Naval Base in San Diego. The San Diego group has been taught our paradigm, given our proprietary software and will test both pure tone sensitivity and emission suppression in a group of seamen both before and after exposure to the firing range for the first time in their military careers.

We have improved the flexibility of our Echo Lab system, developed norms for tone bursts and narrow bands of noise, and completed age and gender norm collection in 60 subjects age groups ranging from 10 to 80. The single pulse paradigm, unavailable on any commercial instrument, was essential here in order to avoid contamination with ipsilateral efferent suppression in which the first

click in a train interferes with the amplitude of the emissions obtained from the subsequent clicks.

We had already made the following important progress at the beginning of this last year:

1. Two versions of our unique and proprietary Lab View system had been built and are operational collecting data in binaural, ipsilateral and contralateral modes in a single-pulse transient-evoked format not otherwise possible.

2. Internal reliability of data collection over a three month period in excess of 0.92.

3. Right vs. Left ear and gender differences are present but only occasionally reach statistical significance (Hood et al. 1996b).

4. Binaural Noise suppresses linear click evoked emissions twice as much as ipsilateral noise and 3 times as much as contralateral noise (Berlin et al., 1995; Appendix 12, 13, 14, 15, 16; confirmed by Liberman et al. 1996). However, subjects with noise exposure often show poor or reduced emissions when the stimulus is a click. Our proprietary system is able to generate transient tone-burst stimuli in singlet form (in contrast to the 4-at-a-time convention which has inherent efferent suppression) and we chose to us a 1500 Hz tone burst after elaborate and extensive pilot testing with various combinations of tones and noises. The basic finding that Binaural noise suppresses more than ipsilateral or contralateral noise in a forward masking paradigm is found again using both the 1500 Hz tone bursts and clicks is as follows in our 30 subjects in whom data are complete:

Table 1 Time Data 8-18 msecs

Mean Suppression updated with 30 Subjects completed as of

September 24, 1997 (s.d. in parentheses)

Note the uneven numbers in cells; some subjects generated usable emission data in only one ear but not the other, despite normal audiograms, and most subjects generated usable tone burst data in one or both ears but many (8) could not produce usable click-emission data.

	Click Stimuli			**1500 Hz. Tone Burst Stimuli**		
	Binaural	Ipsilateral	Contralat.	Binaural	Ipsilateral	Contralat.
Normal Hearing Males Right Ear	-3.867 (1.424) n=11	-1.877 (1.067)	-1.394 (1.078)	-1.422 (0.710) n=16	-0.659 (0.439)	-0.248 (0.707)
Left Ear	-4.096 (2.408) n=10	-1.902 (0.820)	-1.607 (1.473)	-1.166 (0.845) n=16	-0.653 (0.802)	-0.355 (0.818)
Hearing Impaired Males Right Ear	-3.718 (1.814) n=5	-1.759 (0.656)	-1.333 (0.597)	-1.354 (0.616) n=8	-.915 (0.879)	-.268 (1.089)
Left Ear	**-2.066 **** (1.001) n=4	-1.333 (0.860)	-1.395 (0.894)	-1.097 (0.971) n=7	-.593 (0.911)	-.068 (0.926)

Normal Hearing Females N=5 Right Ear	-4.835 n=1, no sd	-2.645	-1.477	-1.116 (0.964) n=5	-.0348 (0.525)	-0.292 (0.297)
Left Ear	-4.099 (3.189) n=3	-1.354 (1.021)	-1.724 (1.390)	-1.189 (1.738) n=5	-0.442 (.821)	-0.450 (.564)

In real-world simultaneous noise conditions the Binaural >ipsi>contra effect is at least three to four times more powerful, but the reader should keep in mind that our data are collected in a forward-masking paradigm in which the masker precedes the onset of the transient by 10 msecs. There is an overall trend for the right ear to show more suppression than the left ear which has appeared periodically in all of our experiments to date but it is most pronounced here in hearing impaired males; however, the uneven subject numbers preclude any meaningful statistical assessment.

5. This pattern of binaural > ipsi > contra suppression is not seen in the few parents we have studied who carry recessive genes for deafness.

6. This Binaural advantage disappeared in the normal hearing 20 adults over age 60 we tested. Thus, suppression and emission strength vary with age, which has to be factored in when one uses emission suppression as a tool for auditory system predictions.

7. The best levels of suppression occur at low levels of noise and when signals are below 65 dB SPL ...the cochlear active process (See summary in: Hood et al. 1996a; Appendix 13).

8. Of 39 subjects enrolled and 30 completely tested so far in the multi-day protocol, subjects with noise exposure effects at higher frequencies show about the same amount of suppression at and around 1500 Hz as subjects with no hearing loss; only the binaural difference to clicks in the left ear may be statistically significant .

9. We also saw a number of our subjects who violated the Binaural>ipsi> contra pattern. This was as common in the hearing loss patients (4 out of 9) as in the normals (10 of 21) and all here but one were under 60.

Liberman and his colleagues have recently elucidated a series of slow vs fast effects in the efferent system which have different effects on the ability of noise to suppress the 2f1-f2 distortion product otoacoustic emission (Sridhar, Liberman, Brown and Sewell, 1995). This slow effect takes about 25 to 50 seconds, in contrast to the fast adaptation effects which are complete in 100 msecs or less. In the guinea pig, fast effects are greatest for mid-frequency components (6-10kHz) while slow effects are seen mostly for high frequency stimuli (12-16 kHz). Similarly, fast effects persist over long periods of time, while slow effects diminish after about a minute of stimulation. Both of these effects can be traced to intracellular mechanisms of the outer hair cell, possibly mediated by a second messenger system.

Reiter and Liberman (1995) postulate that protection from acoustic overstimulation is related to the recently discovered "slow effect" which can only be maintained for 1 to 2 minutes after

overstimulation. Since all of our experiments to date have been clicks and brief tone bursts, it has been impossible for us to see any of Liberman's effects in our human patients. This will be one of our next goals if we have a chance to collect similar data with DPOAE's.

Since Kujawa and Liberman (1997) have shown that total loss of the OCB significantly increases the vulnerability to acoustic damage, we feel the evidence is strong that further studies on human efferent suppression may uncover some natural mechanisms of protection from noise trauma.

8. CONCLUSIONS (PLANS FOR YEAR 05 OF SUPPORT):

ANIMAL PROJECTS: The completed research indicates that ATP is a major neuromodulator in the cochlea, possibly released from the efferent nerve fibers innervating the cells in the cochlea. Furthermore it appears that ATP plays an integral role in cochlea mechanics, in the response of the cochlea to low level sound and toughening, and in noise induced hearing loss. During year #05, we will continue to document the effects of ATP and ATP antagonists on cochlear mechanics and the changes in cochlear mechanics induced by continuous low level sound. We will explore if these ATP induced changes in cochlear mechanics are related to toughening. We will ask if the cochlear mechanics are changed during toughening in a manner that involves ATP receptors. ATP antagonists and newly developed antisense oligos against the ATP receptor mRNAwill be tested against noise induced cochlear damage. In addition, we will continue our studies that indicate ATP administered to short term OHC cultures and into the cochlea induces cell death. ATP antagonists will be used to attempt to block this cell death.

HUMAN PROJECT: For the time being, pending additional funding and support

for our projects, we will be completing data analysis on our nearly 100 subjects and collaborating with Dr. Wester to broaden and complete the prospective study on Navy recruits in San Diego which we hope to pursue in some form of renewal. The lower suppression score of the left ear in response to clicks is a provocative finding which might, in the long run, shed light on the commonly observed phenomenon of left ears having more noise induced trauma than right ears in males. This asymmetry has been ascribed to handedness in rifle and shotgun fire, where the right shoulder protects the right ear from muzzle blast, while the left ear remains open and unprotected. It may ultimately be traceable to a slow effect differential between ears.

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13. Inventions and Patents

None

14. List of Appendices

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Appendix 2. Kujawa, S.G., Fallon, M., and Bobbin, R.P. Time-varying alterations in the f_2-f_1 DPOAE response to continuous primary stimulation. I. Response characterization and contribution of the olivocochlear efferents. Hear. Res. 85: 142-154, 1995.

Appendix 3. Kujawa, S.G., Fallon, M., Skellett, R.A., and Bobbin, R.P. Time-varying alterations in the f_2-f_1 DPOAE response to continuous primary stimulation. II. Influence of local calcium-dependent mechanisms. Hear. Res. 97: 153-164, 1996.

Appendix 4. Bobbin, R.P.M. Chemical receptors on outer hair cells and their molecular mechanisms. In: **Hair Cells and Hearing Aids**. C.I. Berlin, Editor, Singular Publishing Group, Inc. San Diego. p. 29-55, 1996.

Appendix 5. Skellett, R.A., Crist, J.R., Fallon, M. and Bobbin, R.P.M. Chronic low-level noise exposure alters distortion product otoacoustic emissions. Hear. Res.98, 68-76, 1996.

Appendix 6. Skellett, R.A., Fallon, M., Cullen, J.K. and Bobbin, R.P.M. "Conditioning" the auditory system with continuous vs. interrupted noise of equal acoustic energy: Is either exposure more protective? Hear. Res. Submitted, 1997.

Appendix 7. Chen, C., LeBlanc, C., and Bobbin, R.P. (1997a) Differences in the distribution of responses to ATP and acetylcholine between outer hair cells of

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Appendix 9. Bobbin, R.P., Chu, S.H.B., Skellett, R.A., Campbell, J. and Fallon, M. (1997b) Cytotoxicity and mitogenicity of adenosine triphosphate in the cochlea. Hear. Res. In Press.

Appendix 10. Chen, C., Skellet, R.A., Fallon, M. and Bobbin, R.P. (1997b) Additional pharmacological evidence that endogenous ATP modulates cochlear mechanics. Hear. Res. Submitted.

Appendix 11. Bobbin, R.P., Chen, C., Nenov, A.P. and Skellett, R.A. (1997a) Transmitters in the cochlea: the quadratic distortion product and its time varying response may reflect the function of ATP in the cochlea. Berlin, C.I. (Ed.), Recent Advances in Basic and Clinical Hearing Science. Singular Press, San Diego, CA., In Press.

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Appendix 20. Skellett, R. A., Cullen , J.K., Fallon, M., and Bobbin, R.P. Differences in the degree of protection provided by interrupted vs. continuous sound condititoning. Abstracts of the 20th Midwinter Research Meeting, Association for Research in Otolaryngology, St. Petersburg Beach, Florida, abstract 771, February 1-6, 1997.

Appendix 21. Bobbin, R.P, Chen, C., Skellett, R.A., and Fallon, M. The ATP antagonist, suramin, prevents the time related decrease of the quadratic (f2-f1) otoacoustic distortion product. Abstracts of the 20th Midwinter Research Meeting, Association for Research in Otolaryngology, St. Petersburg Beach, Florida, abstract 54, February 1-6, 1997.

Appendix 22. Chu, S.H.B., Fallon, M., Skellett, R.A., Campbell, J., LeBlanc, C.S., and Bobbin, R.P. Adenosine triphosphate (ATP) induces outer hair cell death. Abstracts of the 20th Midwinter Research Meeting, Association for Research in Otolaryngology, St. Petersburg Beach, Florida, abstract 294, February 1-6, 1997.

Appendix #1
DAMD17-93-V-3013
Bobbin and Berlin

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Hearing Research 88 (1995) 215–221

HEARING RESEARCH

Noise exposure alters the response of outer hair cells to ATP

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Received 8 March 1995; revised 9 May 1995; accepted 16 May 1995

Abstract

The outer hair cells (OHCs) are one target of noise-induced effects. To date there are few studies which examine changes in the function of OHCs induced by noise exposure. There is increasing evidence that ATP may be a neuromodulator acting on OHCs. Therefore, we examined the possibility that the response to ATP may be altered by low-level noise exposure. ATP was tested on cation currents recorded from outer hair cells (OHCs) isolated from chronic noise-exposed guinea pigs and compared to currents recorded from normal control animals. The whole-cell variant of the patch-clamp technique was used. The incidence of response to 100 μM ATP was decreased in OHCs from noise-exposed animals as compared to controls when normal internal and external solutions were employed. When K^+ was substituted by *N*-methyl-glucamine (NMG^+) in the pipette solution, there were significant differences in the magnitudes of ATP-evoked currents between cells from noise-exposed and control animals. This was observed in both normal and 20 mM Ba^{2+} external solutions. In addition, the response to ATP exhibited a dependency on OHC length. In short OHCs (< 65 μm) from noise-exposed animals the magnitude of the response to ATP was significantly reduced. By contrast, the response in long OHCs (> 65 μm) from noise-exposed animals was increased. Results suggest that low-level noise exposure induces changes in OHCs which affect the response of the cell to ATP.

Keywords: Voltage-clamp; Patch-clamp; ATP-gated channel; Cochlea; Noise exposure

1. Introduction

Noise exposure induces several alterations in the structure and function of the cochlea (see review by Saunders et al., 1985). The outer hair cells (OHCs) are one target of noise-induced effects (e.g., Cody and Russell, 1985; Puel et al., 1988; Decory et al., 1991; Franklin et al., 1991; Boettcher et al., 1992; Subramaniam et al., 1994). Morphological evidence shows that noise exposure causes: damage to OHC stereocilia, changes in intracellular structures such as mitochondria, and swelling or loss of the OHCs (Saunders et al., 1985). Some of these morphological changes have been correlated with changes in neuronal thresholds (Boettcher et al., 1992) and distortion product otoacoustic emissions (DPOAEs). The latter are thought to reflect the physiological state of the OHCs (Subramaniam et al., 1994).

There are only a few studies which examined the effect of intense sound on the function of the hair cells directly. Decory et al. (1991) showed that isolated OHCs taken from noise-exposed guinea pigs exhibited altered motility and viability. Acoustical-evoked receptor potentials recorded from both inner hair cells (IHCs) and OHCs are altered during and after exposure to intense sound with the OHCs undergoing a sustained depolarization (Cody and Russell, 1985). Some of these effects are probably due to excessive passage of K^+ and Ca^{2+} through the transduction channels of the hair cells together with entrance of Ca^{2+} and Na^+ through voltage-dependent channels. In other systems, it is well known that an increase in the levels of intracellular free Ca^{2+} can initiate events which result in the alteration or death of cells (Berridge, 1994; Morley et al., 1994). Thus there is evidence that the OHCs are excessively depolarized by noise exposure, resulting in altered function and possibly cell death.

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SSDI 0378-5955(95)00115-8

There are a host of molecular events occurring in and around OHCs that could be altered by noise exposure. One possibility is an altered response to neurotransmitter or neuromodulator. Bobbin and Thompson (1978) first proposed ATP as a neuromodulator or transmitter in the mammalian cochlea (see review Eybalin, 1993). Aubert et al. (1994,1995) present data suggesting that ATP may act as a neuromodulator in vestibular organs. ATP and analogues applied into the perilymph compartment exert profound effects on cochlear function, as indicated by an abolishment of compound action potential of the auditory nerve and DPOAEs together with a shift in the summating potential (Bobbin and Thompson, 1978; Kujawa et al., 1994a). Incubation of organ of Corti with ATP agonist analogs increases inositol phosphate accumulation suggesting an activation of this second-messenger system (Niedzielski and Schacht, 1992). Extracellular application of ATP to isolated OHCs of guinea pig depolarizes the cell membrane by inducing non-selective cation currents (Ashmore and Ohmori, 1990; Nakagawa et al., 1990; Housley et al., 1992; Kujawa et al., 1994a; Nilles et al., 1994). Since antagonists also have profound effects on the cochlear potentials, it appears that endogenous ATP may have a role in ongoing physiological mechanisms (Kujawa et al., 1994b). In addition, noise exposure may increase extracellular levels of ATP to such an extent that it contributes to the excessive depolarization and cell death observed.

Thus we initiated studies of the hypothesis that ATP is involved in noise-induced changes to the OHCs. Specifically, in this study we examined whether the response of OHCs to ATP was altered by a low-level, chronic noise exposure. The intensity of the noise was set below the level thought to induce cellular damage (Bohne, 1976). We recorded the cation currents evoked by ATP in isolated OHCs harvested from chronic noise-exposed guinea pigs and compared them to the currents evoked from control animals using the whole-cell configuration of the patch-clamp technique.

2. Methods

2.1. Noise exposure

Guinea pigs ($n = 35$, in groups of 10 or less; age 1–3 months) were placed in a small sound-attenuating booth and exposed to a continuous, moderate-intensity narrow band noise (cutoffs at 1.1 and 2.0 kHz; A-scale, rms: 65 dB SPL) 24 h a day for 10–11 days. Control guinea pigs ($n = 30$) were maintained in an environment of normal noise at the university's animal care facilities. Both groups of the animals were given free access to food and water during the exposure. The care and use of the animals reported on in this study were approved by the Animal Care and Use Committees of the Louisiana State University Medical Center.

Table 1
Composition of solutions (mM)

	Internal		External	
	K^+ sol.	NMG^+ sol.	HBS sol.	Ba^{2+} sol.
NaCl	-	-	137	120
KCl	134	-	5.4	-
$CaCl_2$	0.1	0.5	2.5	-
$BaCl_2$	-	-	-	20
$MgCl_2$	0.5	-	0.5	0.5
CsCl	-	-	-	5
HEPES	5	10	10	10
NMG^+	-	120	-	-
TEA-Cl	-	35	-	-
EGTA	11	11	-	-
Glucose	-	-	10	5
Na_2ATP	2	4	-	-
Na_2GTP	0.1	0.1	-	-
Sucrose	-	10	10	-

HBS: modified Hank's balanced saline; NMG^+: *N*-methyl-glucamine.

2.2. Cell isolation

OHCs from control and noise exposed guinea pigs were acutely isolated as described previously (Erostegui et al., 1994). Briefly, guinea pigs were anesthetized with pentobarbital (30 mg/kg, i.p.) or urethane (1.5 g/kg, i.p.), decapitated, and the bulla separated and placed in a modified Hank's balanced saline (HBS) (Table 1). The bone surrounding the cochlea was removed and the organ of Corti was placed in a 200 μl drop of HBS containing collagenase (1 mg/ml, Type IV, Sigma) for 5 min. The cells were then transferred into the dishes containing a 100 μl drop of HBS using a microsyringe, and stored at room temperature until use (within about 4 h). The length of each cell was measured with a calibrated reticulum prior to recording. OHCs were selected for study if they met several morphological criteria (Ricci et al., 1994). No morphological changes of isolated OHCs from noise-exposed animals were observed.

2.3. Whole-cell voltage clamp

Single dispersed OHCs either from noise-exposed guinea pigs or normal control animals were voltage clamped using the whole-cell variant of the patch-clamp technique (Hamill et al., 1981) with Axopatch-1D and Axopatch-200A patch-clamp amplifiers (Axon Instruments). Patch electrodes were fabricated from borosilicated capillary tubing (Longreach Scientific Resources) using a micropipette puller (Sutter Instrument), and fire polished on a microforge (Narashige Scientific Instrument Lab.) prior to use. Membrane currents were filtered at 5 kHz (−3dB) using a 4-pole low-pass Bessel filter digitized with a 12-bit A/D converter (DMA Interface, Axon Instruments), and stored for off-line analysis using personal microcomputers. Voltage paradigms were generated from a 12-bit D/A converter (DMA Interface, Axon Instruments)

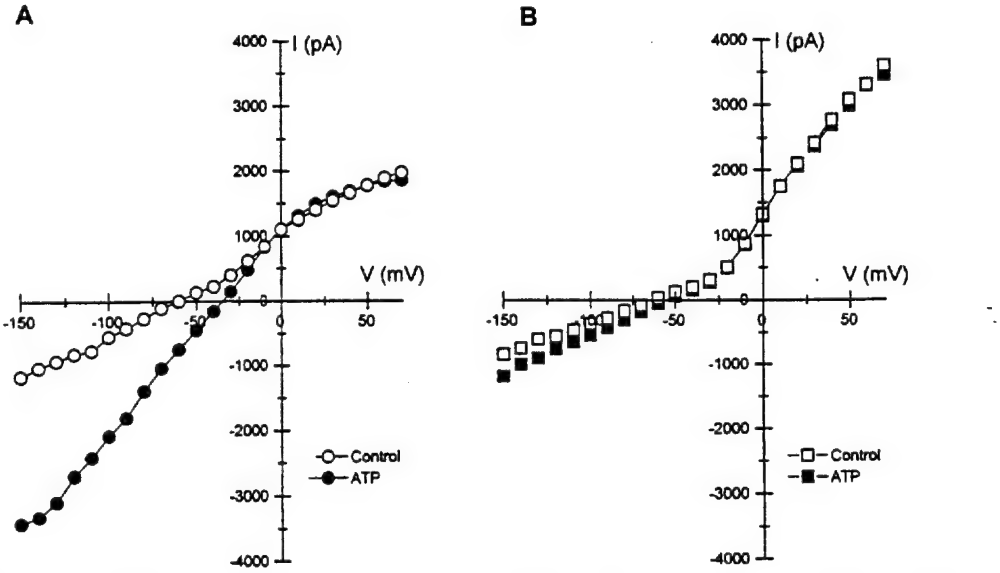

Fig. 1. Typical examples of ATP-induced effects on *I-V* relationships recorded from an OHC taken from a control (A) and noise-exposed animal (B). Currents were evoked by 60 ms steps from a holding potential of -60 mV. Normal HBS external solutions together with K^+-containing internal solutions were used. Currents were measured before (○, □) and during (●, ■) application of 100 μM ATP.

using pClamp software (Axon Instruments). After establishment of the whole-cell configuration, series resistance and cell capacitance compensation were carried out prior to recording, and an 80% series resistance compensation was normally applied. No substraction of leakage current was made.

2.4. Solutions

The composition of the solutions used is shown in Table 1. The HBS solution was utilized for the bath perfusion. ATP (Sigma)-containing external solutions were prepared daily from 100 mM stock solution and was

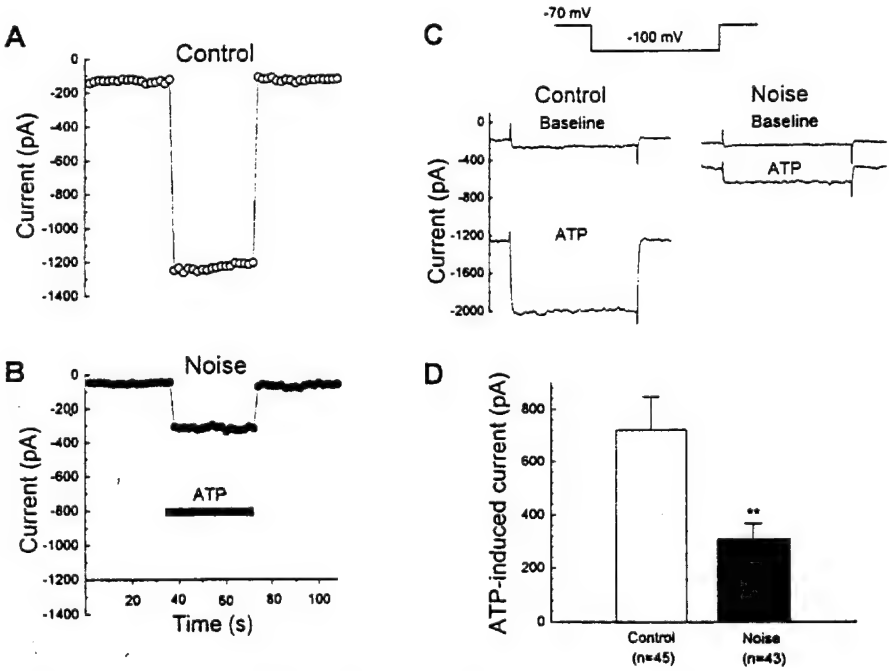

Fig. 2. A and B: typical examples of ATP (100 μM)-induced inward currents recorded from an OHC taken from a control (A) and noise-exposed animal (B). A holding potential of -70 mV and normal HBS external solution together with NMG^+-containing internal solution were used. C: superimposed examples of inward current traces recorded from an OHC taken from a control and a noise-exposed animal in the absence and presence of 100 μM ATP. Current was elicited by a 70 ms hyperpolarizing step to -100 mV from a holding potential of -70 mV. D: amplitudes (mean + SE) of ATP (100 μM)-induced inward currents obtained from OHCs taken from control and noise-exposed guinea pigs. Currents were measured at -100 mV using the step protocol in (C). Statistical significance was measured by ANOVA (** $P < 0.01$).

delivered from an U-tubing system as described previously (Erostegui et al., 1994). All the external solutions were adjusted to a pH of 7.40 with NaOH and had a osmolality of 300 mosM. The internal solutions were adjusted to a pH of 7.35 with HCl and had a osmolality of 284 mosM. All experiments were conducted at room temperature (22 ~ 24°C).

Data are presented as means ± SE. Statistical significance was measured by chi-square test and analysis of variance (ANOVA), as appropriate. P values less than 0.05 were considered statistically significant.

3. Results

Fig. 1 illustrates the current–voltage (I-V) relationships in the absence and presence of 100 μM ATP in OHCs from control and noise-exposed guinea pigs using normal K^+ internal and HBS external solutions. Currents were evoked by 60 ms steps from a holding potential of −60 mV. Monitored at −100 mV, the incidence of ATP responses was significantly reduced in cells from noise-exposed animals as compared to the incidence in cells from control animals (Table 2). In addition, the amplitude of the ATP induced inward current at −100 mV in cells from noise-exposed animals showed a tendency to be smaller than those recorded from control animals ($P = 0.06$).

Table 2
Incidence of response to ATP in OHCs from control and noise-exposed guinea pigs

Control		%	Noise-exposed		%
Response *	No Response *		Response *	No Response *	
25	22	53	14	62	18 **

* Number of cells are given.
** Statistical significance was measured by chi-square test ($P < 0.001$).

To further examine this tendency, we isolated the ATP-induced currents by substituting N-methyl-glucamine (NMG^+) for K^+ in the pipette to block K^+ currents while using normal HBS external solution. Under such conditions, the incidence of the response to 100 μM ATP was not significantly different in the two groups of cells (noise: 88%, $n = 49$ vs. control: 98%, $n = 46$). However, the magnitude of 100 μM ATP-induced inward currents in cells from noise-exposed animals was decreased both at the holding potential of −70 mV (Fig. 2A, control; Fig. 2B, noise) and at the step to −100 mV (Fig. 2C control and noise). Examination of the data at −100 mV indicated that the effect was significant (noise: 303 ± 64 pA, $n = 43$ vs. control: 720 ± 125 pA, $n = 45$; $P < 0.01$; the zero change in response to ATP of the non-responders were not included; Fig. 2D).

To avoid the deleterious effects of increased intra-

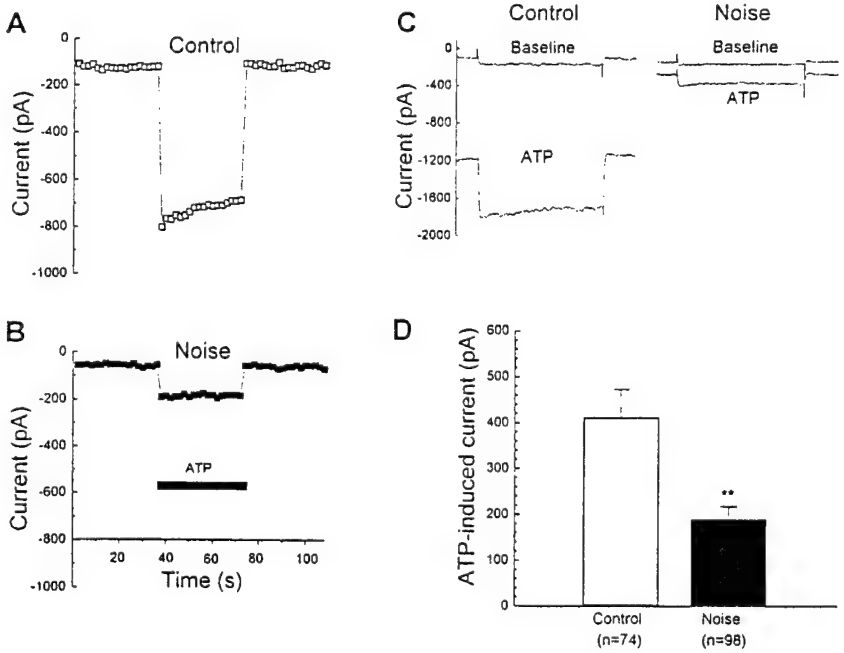

Fig. 3. ATP-evoked current response obtained in external Ba^{2+} solution. A and B: typical examples of ATP (100 μM)-induced inward currents recorded from an OHC taken from a control (A) and noise-exposed animal (B). A holding potential of -70 mV and 20 mM Ba^{2+}-containing external solution together with NMG^+-containing internal solution were used. C: superimposed examples of inward current traces recorded from an OHC taken from a control and noise-exposed animal in the absence and presence of 100 μM ATP. Current was elicited by a 70 ms hyperpolarizing step to -100 mV from a holding potential of -70 mV. D: amplitudes (mean + SE) of ATP-induced inward currents obtained from OHCs taken from control and noise-exposed guinea pigs. Currents were measured at -100 mV using the step protocol in C. Statistical significance was measured by ANOVA (** $P < 0.01$).

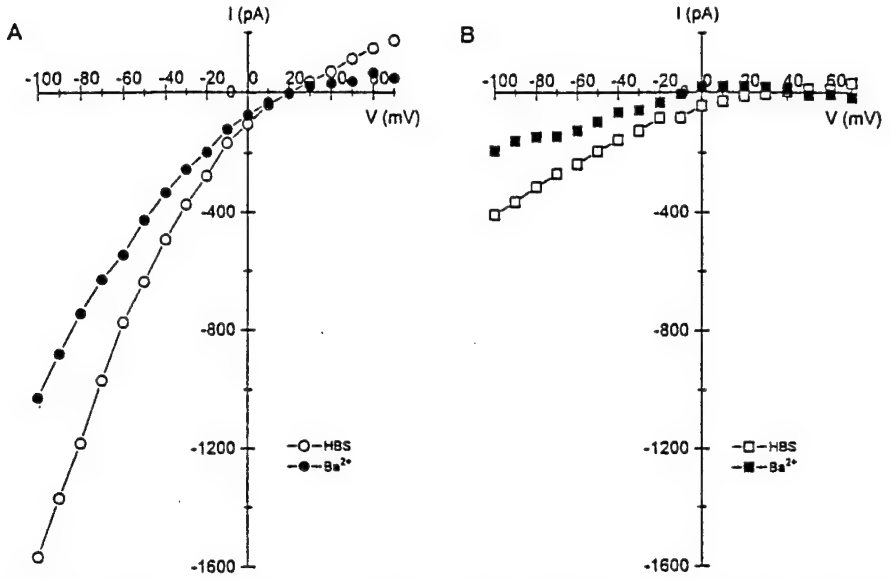

Fig. 4. ATP-evoked current at different voltages. Typical examples of 100 μM ATP-induced effects on *I-V* relationships recorded from an OHC taken from a control (A) and noise-exposed (B) animal. Data shown were obtained utilizing normal HBS external solutions (□), 20 mM Ba^{2+}-containing external solutions (■) and pipettes containing a NMG^{+}-containing internal solution. Plotted are the differences between currents in the presence and absence of the drug at the various test potentials. Currents were evoked by 70 ms steps from a holding potential of -70 mV.

cellular Ca^{2+} caused by ATP and to further block outward K^{+} current, we studied the effect of utilizing Ba^{2+}-containing external solution while keeping the same NMG^{+}-containing solution in the pipette. Some of the cells ($n = 26$, noise; $n = 27$, control) were the same ones studied in the previous paragraph. Results were similar to that using

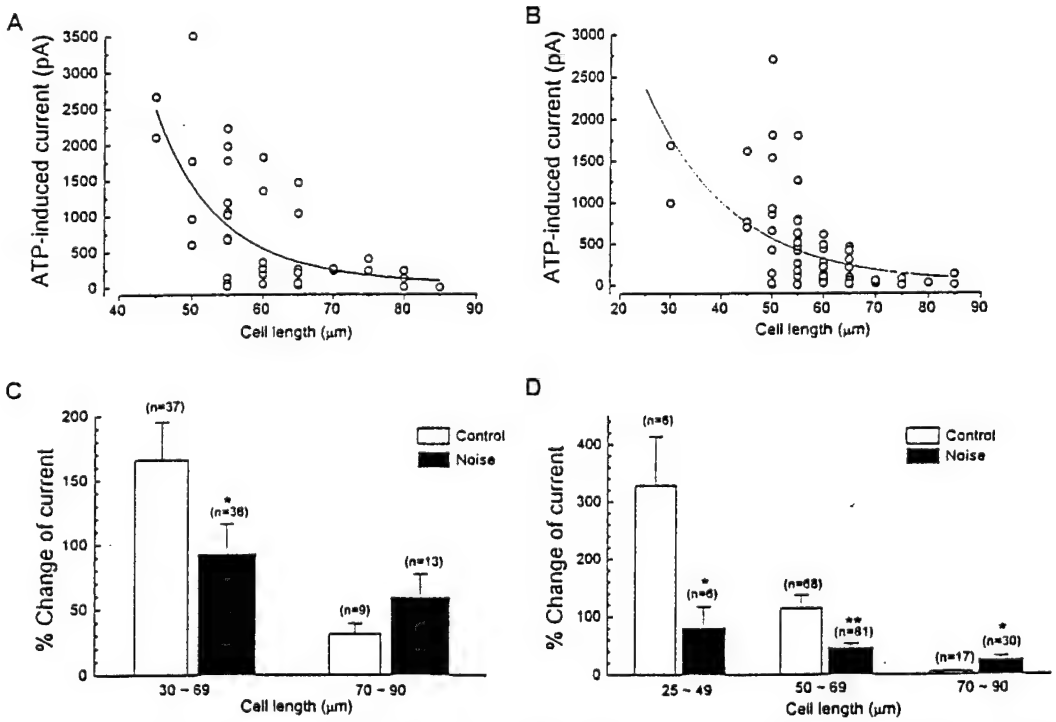

Fig. 5. A: scattergram of the 100 μM ATP-induced currents recorded from OHCs taken from control animals plotted against cell length. Data obtained utilizing normal HBS external solutions together with NMG^{+}-containing internal solutions. B: same as in (A) only data obtained utilizing 20 mM Ba^{2+}-containing external solutions together with NMG^{+}-containing internal solutions. C: comparison of the magnitude of responses to 100 μM ATP between OHCs taken from control and noise-exposed guinea pigs. Data obtained utilizing normal HBS external solutions together with NMG^{+}-containing internal solutions. D: same as in (C) only utilizing 20 mM Ba^{2+}-containing external solutions together with NMG^{+}-containing internal solutions. The divisions according to length were arbitrarily made. In some cases the same cell contributed data that appears in both normal and 20 mM Ba^{2+}-containing external solution groups. Each bar is mean + SE. Statistical significance was measured by ANOVA (* $P < 0.05$; ** $P < 0.01$). All current amplitudes were measured at -100 mV using a step protocol consisting of 70 ms hyperpolarizing steps to -100 mV from a holding potential of -70 mV.

HBS external solution. There was no difference between groups in the incidence of the response (noise: 84%, $n = 117$ vs. control: 81%, $n = 91$). However, the magnitude of the ATP-induced currents in cells from noise-exposed animals was decreased both at the holding potential of −70 mV (Fig. 3A, control; Fig. 3B, noise) and at the step to −100 mV (Fig. 3C, control and noise). Examination of the data at −100 mV indicated that the response was significantly different from control (noise: 188 ± 29 pA, $n = 98$ vs. control: 410 ± 62 pA, $n = 74$; $P < 0.01$; Fig. 3D). Fig. 4 shows the *I-V* relationships of ATP-induced inward currents in OHCs from control (Fig. 4A) and noise-exposed (Fig. 4B) animals in HBS and Ba^{2+}-containing external solutions. The amplitudes of the ATP-elicited inward currents were smaller in Ba^{2+}-containing external solution than those in HBS solution. The pattern of results in OHCs from control and noise-exposed animals were similar.

Length of OHCs varies according to their position in the cochlear partition and is related to the function of OHCs (Pujol et al., 1992). We observed a length dependency for the ATP-induced effects in HBS (Fig. 5A) and Ba^{2+}-containing (Fig. 5B) external solutions for OHCs taken from control animals. This result is similar to that reported for acetylcholine-induced effects in OHCs (Erostegui et al., 1994). Fig. 5C (in HBS solution) and Fig. 5D (in Ba^{2+}-containing solution) illustrate the amplitudes of ATP-induced inward currents expressed as a percent of baseline currents (at −100 mV) in cells grouped according to length. The amplitudes of the currents were significantly decreased in the short cells (cell length < 65 μm) from noise-exposed animals as compared to those from controls. In contrast, ATP-induced currents were enhanced in cells with lengths over 65 μm from noise-exposed animals as compared to corresponding cells from controls.

4. Discussion

Results show that there were significant differences in the incidence and magnitudes of cation currents evoked by ATP (100 μM) in OHCs taken from guinea pigs exposed to chronic low-level noise when compared to the currents evoked from cells obtained from control animals. The response to ATP was decreased in short OHCs whereas in longer OHCs the response was increased.

At present we can only speculate as to the structural or biochemical mechanisms underlying our observations. The results are in harmony with the hypothesis that endogenously released ATP may be involved in the effects induced in the cochlea by low-level, chronic noise exposure. In other systems, the chronic release of ATP has been suggested to result in a down-regulation of purinoceptors (Maynard et al., 1992). This mechanism was proposed to explain the altered response to an ATP analogue in the rabbit isolated central ear artery after chronic electrical stimulation of the great auricular nerve (Maynard et al., 1992). By analogy, during noise exposure there may be a continuous exposure of OHCs to a high level of endogenous ATP which induces a similar down-regulation of ATP receptors in short OHCs. An up-regulation of ATP receptors may occur in long OHCs. Only future experiments can determine if such a mechanism accounts for the observed altered responses to ATP.

Alternatively, any noise-induced structural damage may have altered the response to ATP. We did not determine whether damage had occurred in the cochlear tissue we sampled. On the other hand, based on the literature to date, the noise level was probably not sufficiently intense to produce physical damage. No reports were found using the same band and level of noise in guinea pigs. However, the noise is the same level as one of the octave band of noise (0.5 kHz) exposures used by Bohne (1976). Bohne showed no damage to chinchilla hair cells at the light microscopic level after 9 days of continuous exposure to the 65 dB SPL noise. Others have shown that the guinea pig is slightly less susceptible to noise damage than the chinchilla (Decory et al., 1992). In other studies utilizing chinchillas, a more intense chronic noise exposure (85 dB SPL) resulted in stereocilia damage (Boettcher et al., 1992; Subramaniam et al., 1994). ATP receptors have been proposed to be located near the stereocilia on the apical surface membrane (Housley et al., 1992; Mockett et al., 1994). Thus the decreased response to ATP may be due to some physical or chemical alteration in the ATP receptors located near or on damaged stereocilia. On the other hand, the increased response observed in long OHCs argues against a physical damage and suggests a more complex mechanism.

Aside from damage, a subject's acute and chronic history of sound exposure changes the response of the cochlea to sound. Acute, low-level sound alters the mechanics of the cochlear partition in a complex manner as monitored by quadratic DPOAEs (Kujawa et al., 1995). Chronic, higher level exposure decreases the effect of subsequent noise exposure (Clark et al., 1987; Sinex et al., 1987; Canlon et al., 1988,1992; Campo et al., 1991; Franklin et al., 1991; Boettcher et al., 1992; Mensh et al., 1993; Subramaniam et al., 1994). During this latter effect which is called 'toughening', changes in the mechanics of the cochlea as monitored with DPOAEs have been reported (Franklin et al., 1991; Mensh et al., 1993; Subramaniam et al., 1994). In both these acute and chronic phenomena, the changes in DPOAEs and cochlear mechanics may indicate alterations in the function of the OHCs, possibly involving ATP. Whether the exposure utilized in the present study alters DPOAEs remains to be determined.

In summary, results indicate that the response to ATP was altered in OHCs obtained from noise exposed guinea pigs. We speculate that this is due to an alteration in the number of ATP receptor proteins. Additional research will be necessary to determine whether this mechanism actually underlies the observed phenomena.

Acknowledgements

Thanks to Maureen Fallon, Ruth Skellett, and Christopher LeBlanc, for their technical help and to Sharon Kujawa for advice. Supported by DAMD 17-93-V-3013, NIH grant R01-DC00722, Kam's Fund for Hearing Research, and the Louisiana Lions Eye Foundation.

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Hearing Research 85 (1995) 142–154

HEARING RESEARCH

Time-varying alterations in the f_2-f_1 DPOAE response to continuous primary stimulation I: Response characterization and contribution of the olivocochlear efferents ☆

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Received 18 October 1994; revised 2 February 1995; accepted 18 February 1995

Abstract

The f_2-f_1 distortion product otoacoustic emission (DPOAE) can be observed to undergo gradual alterations in amplitude during continuous ipsilateral stimulation with primary tones. In the present experiments, we characterized the dependence of these amplitude alterations on several stimulus variables (intensity, duration, frequency) and on DPOAE type (quadratic vs cubic) and tested the hypothesis that such alterations are mediated by the olivocochlear (OC) efferents. Responses were recorded in urethane-anesthetized guinea pigs with sectioned middle ear muscles before and after intracochlear application of antagonists (curare, 1 μM; bicuculline, 10 μM; tetrodotoxin, 1 μM) or before and after OC efferent section at the midline of the floor of the IVth ventricle. We confirm previous reports of continuous stimulation-related alterations in the amplitude of the quadratic distortion product, f_2-f_1, and report a novel, suppressive 'off-effect' apparent in f_2-f_1 amplitude following a short rest from such stimulation. Response alterations were sensitive to primary intensity and to duration of rest from continuous stimulation, but were not clearly frequency-dependent over the ranges tested. Corresponding alterations in the amplitude of the cubic nonlinearity, $2f_1-f_2$ were very small or absent. Amplitude alterations in f_2-f_1 were reduced but not blocked by OC efferent antagonists (curare, bicuculline) and were largely unaffected by TTX or by midline brainstem section. All of these manipulations, however, prevented completely the known efferent-mediated contralateral sound suppression of both f_2-f_1 and $2f_1-f_2$ DPOAEs. Taken together, these results do not provide support for efferent control of the f_2-f_1 amplitude alterations observed during continuous ipsilateral stimulation.

Keywords: Olivocochlear efferents; Otoacoustic emissions; Outer hair cells; Quadratic nonlinearity; Cubic nonlinearity

1. Introduction

The quadratic nonlinearity, f_2-f_1, measured as a distortion product otoacoustic emission (DPOAE), displays time-varying amplitude alterations during continuous, primary tone stimulation. Brown (1988) first described a short period of amplitude growth followed by large (often exceeding 15 dB) reductions in f_2-f_1 DPOAE amplitude during continuous low- to moderate-level primary stimulation. Similar amplitude alterations were not observed in the cubic nonlinearity corresponding to the frequency $2f_1-f_2$. In a subsequent investigation, Whitehead et al. (1991) also observed f_2-f_1 but not $2f_1-f_2$ to undergo an initial amplitude increase followed by a gradual decline during continuous primary stimulation. Rarely exceeding 3 dB, the magnitude of the f_2-f_1 amplitude reduction was much smaller than that observed by Brown. Kirk and Johnstone (1993) confirmed the perstimulatory reductions in f_2-f_1 amplitude and reported effects to be most robust within the primary frequency range 2–7 kHz. Again, corresponding changes in the $2f_1-f_2$ distortion product were minimal or absent at any of the frequency combinations tested.

☆ A preliminary report was presented at the 127th Meeting of the Acoustical Society of America in June, 1994.

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SSDI 0378-5955(95)00041-0

In contrast to the different behavior of these distor-
ɔn components during ipsilateral stimulation, both
$-f_1$ and $2f_1-f_2$ distortion products can be altered
ıring presentation of tones or noise to the contralat-
al ear (e.g., Kirk and Johnstone, 1993; Kujawa et al.,
)93, Kujawa et al., 1994; Puel and Rebillard, 1990).
'hen appropriate measures are taken to avoid tran-
ranial crossover of the contralateral signal or middle
ır muscle activation, these contralateral influences on
silateral DPOAEs reveal clearly an efferent influence
ı the cochlear mechanics. This efferent control is
ought to be mediated primarily by uncrossed medial
ivocochlear (UMOC) neurons which synapse directly
ith outer hair cells (OHCs) and which, due to the
edominantly crossed nature of the afferent pathways,
e activated primarily by contralateral sound (Warren
ıd Liberman, 1989). Such response suppression can
e mimicked by electrical activation of OC neurons
Kirk and Johnstone, 1993; Mountain, 1980; Siegel and
im, 1982). It can be prevented by cuts to the brain-
em that remove this efferent input (Puel and Rebil-
rd, 1990; Puria et al., 1992) and it can be blocked
ıarmacologically by antagonists of OC efferent activ-
y (Kujawa et al., 1993; Kujawa et al., 1994).

The mechanisms underlying the f_2-f_1 amplitude
terations during continuous ipsilateral stimulation are
ıknown. Brown (1988) and Kirk and Johnstone (1993)
ıve presented results suggesting that the ipsilateral
'fects, too, are under efferent control. In the Brown
988) experiments, f_2-f_1 response alterations were
)sent or reduced in magnitude in deeply-anesthetized
ıimals. Additionally, they were altered by the ipsilat-
al presentation of 'novel' stimuli and by stimulation
the contralateral ear during periods of rest from
silateral stimulation. In the Kirk and Johnstone (1993)
udies, the amplitude alterations were prevented in
)me animals by intracochlear application of bicu-
ılline, an antagonist of GABA, a putative transmitter
apical cochlear efferents (see Eybalin, 1993 for
view) and by tetrodotoxin (TTX). The amplitude
terations were not, however, blocked by strychnine,
hich has potently antagonized every known OC effer-
ıt-mediated effect identified to date (e.g., Bobbin and
onishi, 1974; Desmedt and Monaco, 1962; Kujawa et
., 1994). Yet, in these same animals, strychnine
ocked contralateral suppression of f_2-f_1 and abol-
hed the suppressive effects of electrical OC stimula-
ɔn on both the compound action potential (CAP) of
e auditory nerve and the f_2-f_1 DPOAE. Moreover,
'hitehead et al. (1991) found no support for efferent
)ntrol of these amplitude alterations in rabbit. Specif-
ally, responses were not different in awake and anes-
etized animals, and they were not altered by con-
alateral sound. Finally, none of these investigations
ıve revealed corresponding alterations in the ampli-
de of the $2f_1-f_2$ DPOAE. Given the similar behav-
ior of the f_2-f_1 and $2f_1-f_2$ DPOAEs in response to efferent activation by contralateral sound, it remains unclear why ipsilateral sound-activation of efferent neurons should affect f_2-f_1 and not $2f_1-f_2$ DPOAEs.

Our ultimate goal in these experiments is to identify the mechanism(s) underlying the f_2-f_1 DPOAE amplitude alterations observed during continuous primary stimulation. In this paper, we confirm the amplitude alterations observed by others and report, in addition, a suppressive 'off-effect' of continuous stimulation not described in previous reports. We describe the dependence of these amplitude alterations on several stimulus variables (level, duration and frequency) and on DPOAE type (quadratic vs cubic). In addition, we present results related to our tests of the hypothesis that the amplitude alterations are under efferent control. Toward this end, we studied two antagonists of OC efferent activity (curare, bicuculline) that we have found previously to block contralateral sound suppression of the $2f_1-f_2$ DPOAE (Kujawa et al., 1994), an antagonist of all action potential-mediated activity (TTX) and OC nerve section for their effects on these time-varying changes in f_2-f_1 amplitude. Contralateral suppression of f_2-f_1 and $2f_1-f_2$ DPOAEs was employed as an internal, known efferent-mediated control for the effects of these experimental manipulations.

2. Methods

2.1. Subjects

Pigmented guinea pigs (250–400 g) of either sex were anesthetized (urethane, 1.5 g/kg, i.p.) and tracheotomized but were allowed to breath unassisted. ECG and rectal temperature were monitored throughout each experiment, and temperature was maintained at 38° ± 1° C. Additional urethane was administered as required to maintain an adequate depth of anesthesia.

Surgical procedures have been described previously (Kujawa et al., 1994). Briefly, cartilaginous ear canals were exposed and partially removed to allow optimum coupling of the sound delivery systems to the two ears. A subgroup of these animals was used in the cochlear perfusion experiments described in this report. Thus, in all animals, the right auditory bulla was exposed and opened ventrally to gain access to the cochlea and tendons of the right middle ear muscles were sectioned.

2.2. Stimulus generation and response monitoring

The DPOAE under primary investigation during continuous ipsilateral stimulation was the f_2-f_1 DPOAE at 1.25 kHz. For certain ipsilateral and all contralateral stimulus conditions, we also monitored the cubic distortion product at the $2f_1-f_2$ frequency (5

kHz). The instrumentation employed in these experiments has been described (Kujawa et al., 1994). Briefly, responses were elicited by equilevel primary stimuli (f_1 = 6.25 kHz; f_2 = 7.5 kHz) generated by oscillators, routed through attenuators to separate speakers and delivered to the right ear of each animal by an acoustic probe assembly. Output from the probe microphone was led, via a microphone preamplifier, to a dynamic signal analyzer for averaging (10 discrete spectra) and display (span = 1 kHz; CF = DP frequency; BW = 3.75 Hz). The noise floors associated with these display windows averaged approximately −15 dB SPL for the f_2–f_1 DPOAE and −18 dB SPL for the $2f_1$–f_2 DPOAE when measured at points ±50 Hz of the distortion product frequency. In some experiments, distortion products (f_2–f_1 and $2f_1$–f_2) to other primary pairs were generated; details pertaining to those stimulus conditions will be discussed where appropriate. For contralateral suppression measures, the contralateral stimulus was a 70 dB SPL overall level wideband noise (WBN; 0.9–15.7 kHz), generated and delivered to the left ear as detailed in previous reports (Kujawa et al., 1993; Kujawa et al., 1994).

2.3. Baseline measures

Baseline measures of f_2–f_1 (1.25 kHz) and $2f_1$–f_2 (5 kHz) distortion product growth with increasing stimulus level (25–70 dB SPL in 5 dB steps) were obtained at the right ear of each animal following surgical exposure of the cochlea and middle ear muscle section. Only those animals whose DPOAE growth functions conformed to laboratory norms following these procedures participated in the experiments that followed. A 15 min period without stimulation separated these measures from the next period of primary stimulation.

The influence of continuous, moderate-level (60 dB SPL) primary stimulation on the f_2–f_1 DPOAE at 1.25 kHz was studied in all animals (N = 35). The following stimulation and response monitoring protocol was employed: 100 consecutive 10-spectra averages of distortion product amplitude were obtained during continuous primary stimulation. Each of these averages required approximately 5 s to complete for a total of 500 s (8.3 min) of stimulation. The primary tones were then simultaneously turned off and there was a 1 min rest from primary stimulation. Following this rest, the primaries were re-introduced and 40 consecutive 10-spectra averages of distortion product amplitude were obtained (total time approximately 200 s or 2.3 min of stimulation). Here again, and for all subsequent stimulus manipulations, a 15 min period of rest from primary stimulation separated each test condition from the next.

Contralateral suppression of both the f_2–f_1 and $2f_1$–f_2 DPOAEs also was studied in all animals. Methods were similar to those employed previously (Kujawa et al., 1994). Briefly, 5 consecutive, 10-spectra averages of DPOAE amplitude (to equilevel primaries at 60 dB SPL) were obtained in the absence of contralateral stimulation. The WBN was then introduced to the contralateral ear and 5 consecutive, 10-spectra averages of distortion product amplitude were obtained during contralateral stimulation. The noise was removed and 5 additional 10-spectra averages of DPOAE amplitude were obtained.

Following these baseline response characterizations, animals were employed in experiments designed to characterize the dependence of these amplitude alterations on the stimulus variables of intensity, duration and frequency and on DPOAE type and/or they served as subjects in the cochlear perfusion or nerve section studies.

2.4. Response characterization experiments

Intensity effects

To investigate the influence of primary level on f_2–f_1 DPOAE amplitude alterations, a subgroup of animals was tested with equilevel primaries at 40, 50 and 70 as well as 60 dB SPL. Responses were monitored as described above.

Duration effects

To clarify the dependence of these time-varying changes in f_2–f_1 DPOAE amplitude on continuous primary stimulation, f_2–f_1 amplitude changes associated with intermittent periods of short-duration (5 s) primary stimulation ($L_1 = L_2$ = 50–70 dB SPL) were studied over the same time period. For these comparisons, single 10-spectra averages were obtained at times corresponding to the first average of the continuous series, the 100th average and the final (40th) average following the return to stimulation. Alternatively, following the initial period of continuous primary stimulation (500 s) and 1 min rest, f_2–f_1 distortion product recovery was tracked using 5 s periods of stimulation obtained at 30 s intervals. Results were compared to those obtained in the same animals using our standard (baseline) protocol.

The influence of rest period duration on amplitude changes in the f_2–f_1 DPOAE as recorded following the first period of continuous stimulation was investigated using primaries at 60 dB SPL. This was accomplished by halving (30 s) or doubling (2 min) the period of rest from continuous primary stimulation and then comparing results to those obtained in the same animals with the standard (1 min) rest.

Frequency effects

The influence of primary frequency was investigated by comparing alterations in the amplitude of the f_2–f_1 DPOAE when generated by lower (f_1 = 2.5 kHz; f_2 = 3

Hz) and by higher ($f_1 = 10$ kHz; $f_2 = 12$ kHz) frequency primaries to results obtained with the standard primary pair ($f_1 = 6.25$ kHz; $f_2 = 7.5$ kHz). For these measurements, the f_2/f_1 ratio was held constant at 1.2 and primaries were equal in level at 60 dB SPL.

DPOAE type

The effect of DPOAE type on the magnitude and the course of the amplitude alterations during continuous primary stimulation was investigated under several separate conditions: First, amplitude alterations in the cubic ($2f_1-f_2$) DPOAE at 5 kHz were compared to those recorded for the quadratic (f_2-f_1) DPOAE at 5 kHz for identical stimulus frequency and intensity conditions ($f_1 = 6.25$ kHz; $f_2 = 7.5$ kHz; $L_1 = L_2 = 60$ dB SPL). Second, a $2f_1-f_2$ DPOAE was elicited at 5 kHz ($f_1 = 1.55$ kHz; $f_2 = 1.86$ kHz; $L_1 = L_2 = 60$ dB SPL) and amplitude alterations observed for this distortion product were compared to those for the f_2-f_1 DPOAE at the same frequency and for the same primary levels. Finally, in an attempt to compare f_2-f_1 and $2f_1-f_2$ DPOAEs similar in baseline amplitude, the $2f_1-f_2$ DPOAE at 5 kHz was elicited by equilevel primaries at 50 dB SPL and response alterations to continuous stimulation under these conditions were compared to those obtained for the f_2-f_1 DPOAE response to higher level (60 dB SPL) primaries.

. Cochlear perfusion experiments

Perfusion experiments were undertaken using methods described previously (Kujawa et al., 1994). The artificial perilymph had a composition of (in mM): Cl, 137; KCl, 5; $CaCl_2$, 2; NaH_2PO_4, 1; $MgCl_2$, 1; glucose, 11; $NaHCO_3$, 12 with a resulting pH of 7.4 when brought into solution in a deionized and filtered water base. Experimental drugs tested for their effects on ipsilateral and contralateral stimulation-related DPOAE amplitude alterations were the following: bicuculline (bicuculline methiodide, 10 μM, Sigma), curare (d-tubocurarine chloride, 1 μM, Sigma) and TTX (tetrodotoxin, 1 μM, Sigma). The agents were dissolved in the artificial perilymph (pH 7.4). Perfusates were introduced into the cochlear perilymph at a rate of 2.5 μl/min for 10 min through a hole in basal turn scala tympani and were allowed to flow from the cochlea through an effluent hole placed in basal turn scala vestibuli. Effluent was absorbed within the bulla using small cotton wicks.

For the purposes of the perfusion experiments, animals were divided into 4 groups: One group of animals ($N = 5$) received 8 consecutive perfusions of the control (artificial perilymph) solution alone. Effects of the MOC efferent antagonists, bicuculline and curare, were tested in another group of animals ($N = 5$). These animals first received 2 perfusions of the control (artificial perilymph) solution alone. These perfusions were followed, in order, by a single perfusion of bicuculline, 2 artificial perilymph washes, a single perfusion of curare and two additional artificial perilymph washes. To insure that drugs were not being diluted with replacement CSF during the long response monitoring periods post-perfusion, a third group of animals ($N = 4$) received these drugs after first undergoing blockade of the cochlear aqueduct as described by Jenison et al. (1985). Effects of TTX were studied in a fourth group of animals ($N = 6$). Again, the first 2 perfusions were of artificial perilymph alone. These perfusions were followed by a single perfusion of TTX followed by 2 artificial perilymph washes. Immediately following each perfusion, measures of f_2-f_1 and $2f_1-f_2$ DPOAE amplitudes before, during and after contralateral WBN stimulation were obtained followed by measures of f_2-f_1 amplitude during continuous ipsilateral stimulation (60 dB SPL) as described for baseline characterizations.

2.6. OC nerve section experiments

The effect of OC section at the brainstem midline on the ipsilateral stimulation-related changes in f_2-f_1 amplitude was studied in $N = 4$ animals. Such a section should effectively remove input from the vast majority of ipsilaterally-responsive (i.e., crossed) MOC neurons to each cochlea (Liberman and Brown, 1986). In these animals, the middle cerebellar vermis was aspirated, revealing the floor of the IVth ventricle. Measures of ipsilateral stimulation-related effects on f_2-f_1 DPOAE amplitude to 60 dB SPL primaries were obtained to identify changes in response characteristics that might be associated with these surgical manipulations. A midline cut spanning the anterior-posterior extent of the exposed IVth ventricle floor at a depth of approximately 2 mm was then made and ipsilateral and contralateral stimulation measures were obtained. Results of both sets of measures were compared to baseline data for these animals.

Effects of treatments on ipsilateral and contralateral stimulation-related changes in DPOAE amplitude were quantified using repeated measures analysis of variance (ANOVA) and Tukey post-hoc tests. The care and use of the animals reported on in this study were approved by LSUMC's Institutional Animal Care and Use Committee.

3. Results

3.1. Amplitude alterations during continuous ipsilateral stimulation

During continuous stimulation with moderate-level (60 dB SPL) primaries, the f_2-f_1 DPOAE was ob-

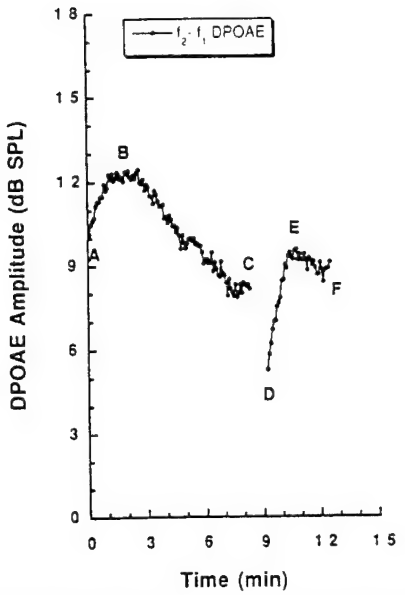

Fig. 1. Effect of continuous primary stimulation on f_2-f_1 DPOAE amplitude ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). Each data point represents a 10 spectra average and required 5 s to complete. Break in response amplitude trace (C–D) represents 1 min with no primary stimulation. Points A–F thus identified in each trace were used to calculate magnitudes of component amplitude changes, slopes of suppression and recovery functions and for statistical analyses across animals.

served to undergo stereotyped amplitude alterations. A representative example is shown in Fig. 1. Following a short period of amplitude growth during the first 2 min of continuous primary stimulation, the f_2-f_1 DPOAE underwent a slow decline which either progressed at a similar rate throughout the remainder of the stimulation period (approximately 6–7 min) as in this example, or which gradually fell to a new, lower level and then changed comparatively little during the last few minutes of stimulation. Following a 1 min rest from continuous primary stimulation, a return to stimulation found the DPOAE further suppressed from its pre-rest amplitude. Thereafter, f_2-f_1 amplitude increased rapidly, again reaching a maximum approximately 2 min into the period of continuous primary stimulation. Periodic checks of primary amplitudes revealed changes less than ± 0.1 dB across similar periods of stimulation.

This general response configuration was obtained for 33 of 35 animals tested in this series. In the remaining 2 animals, the post-rest amplitude suppression was absent, but all other amplitude alterations followed the characteristic pattern. Six points (A–F) were identified on each response amplitude function (see Fig. 1). These values were used to calculate the magnitudes of the various amplitude changes, the overall slopes of the suppression and recovery functions and for statistical analyses. The period of amplitude growth observed following stimulus onset will be referred to as an 'on-effect' of continuous primary stimulation. The first such on-effect is represented as the change in amplitude from point A (DPOAE amplitude at the first 10 spectra average) to point B (the 10 spectra average yielding the maximum DPOAE amplitude during the first period of continuous stimulation). In the 35 animals tested with equilevel primaries at 60 dB SPL, this amplitude increase averaged 1.52 ± 0.14 dB at its peak (B), which occurred 1 min 44 s ± 6 s into the period of continuous primary stimulation (A to B slope = 0.88 dB/min). After reaching this amplitude maximum, the response declined slowly during the remainder of the stimulation period (B to C slope = -0.42 dB/min). At the end of this period of continuous primary stimulation (C), the amplitude of the distortion product was reduced 1.24 ± 0.31 dB from its amplitude at onset (A). However, it was reduced from its peak value (B) by 2.75 ± 0.25 dB. At the return to stimulation (D) following a 1 min rest, the f_2-f_1 DPOAE was further suppressed from this pre-rest value (C) by 1.26 ± 0.12 dB. Of interest, the magnitude of the amplitude decline occurring during this 1 min rest from continuous stimulation is 3 times that observed for a comparable time period during which the primaries were delivered continuously. This post-rest amplitude suppression (the change in DPOAE amplitude from point C to point D) will be referred to as the 'off-effect'. Following the return to stimulation, response amplitude increased rapidly (2.44 ± 0.18 dB at point E). This second on-effect (ΔD–E) demonstrated a time course similar to the first, reaching a new maximum 2 min 2 s ± 7 s into the second period of continuous stimulation (D to E slope = 1.20 dB/min). After reaching this amplitude maximum, the response again began on a downward course, clearly evident at point F (E to F slope = -0.43 dB/min). All amplitude changes from baseline reached significance ($P < 0.01$).

When monitored over repeated trials in the same animal, these characteristic f_2-f_1 amplitude alterations demonstrated little variability. Fig. 2 displays the results of repeated averages separated by periods of

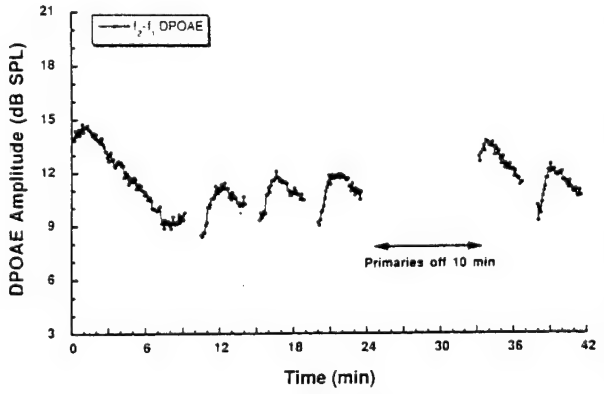

Fig. 2. Effect of repeated primary stimulations separated by 1 min or 10 min rest periods on f_2-f_1 DPOAE amplitude ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL).

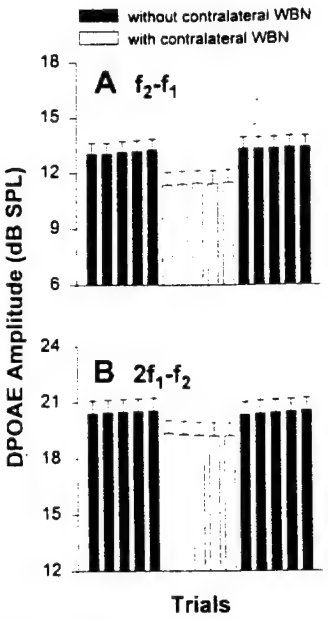

Fig. 3. Effect of contralateral WBN on (A) f_2-f_1 and (B) $2f_1-f_2$ DPOAE amplitude ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). DPOAEs were monitored before (5 trials, solid bars), during (5 trials, open bars) and after (5 trials, solid bars) presentation of WBN (70 dB SPL) to the contralateral ear. Each 'trial' represents a 10 spectra average and required 5 sec to complete. Data are represented as means ± S.E. (N = 35).

rest (1 min; 10 min) for one representative animal. Although the extent of the amplitude decline is limited in subsequent trials (due to the shortened periods of continuous stimulation; approximately 3.3 vs 8.3 min), on- and off-effects are nearly identical in magnitude and time course and the slopes of the growth and decay portions of the curves remain constant. After a longer period of rest from continuous stimulation (10 min), some additional recovery of distortion product amplitude is evident. Similar results were obtained in 4 additional animals.

3.2. Amplitude alterations during contralateral WBN stimulation

Contralateral suppression of both f_2-f_1 and $2f_1-f_2$ DPOAEs was observed in 34 of 35 animals tested. The f_2-f_1 DPOAE was reduced approximately 1.7 dB (Fig. 3A) and the $2f_1-f_2$ DPOAE was reduced approximately 1.2 dB (Fig. 3B) in the presence of a 70 dB SPL contralaterally-presented WBN. In both cases, the contralateral stimulation-associated reductions in DPOAE amplitude reached significance ($P < 0.001$). When contralateral sound effects are expressed in terms of percent reduction of baseline distortion product amplitude, the f_2-f_1 DPOAE was reduced by roughly 13%, the $2f_1-f_2$ DPOAE by 6%.

3.3. Effects of stimulus variables and DPOAE type

Intensity effects

On average ($N = 9$), the magnitudes of the component amplitude alterations associated with continuous ipsilateral stimulation decreased as stimulus intensity was increased (Figs. 4A–C). Intensity-related changes in on- (ΔA–B; ΔD–E) and off-effect magnitudes (ΔC–D) reached overall significance, with post-hoc comparisons (Tukey) revealing significant differences between values obtained at 50 and 60 dB SPL ($P < 0.01$) and 50 and 70 dB SPL ($P < 0.01$) but not 60 and 70 dB SPL ($P > 0.05$). The magnitude of the pre-rest amplitude decline (ΔB–C) associated with 50 dB SPL primaries was more variable between animals. Thus, intensity-related alterations in the magnitude of pre-rest amplitude decline failed to reach overall significance ($P > 0.05$). Responses to primary stimuli at 40 dB SPL ($N = 3$ animals) were very small and were more variable in amplitude over repeated averages within animals. It could not, therefore, reliably be determined whether effects of continuous stimulation continued to grow as primary level was reduced further.

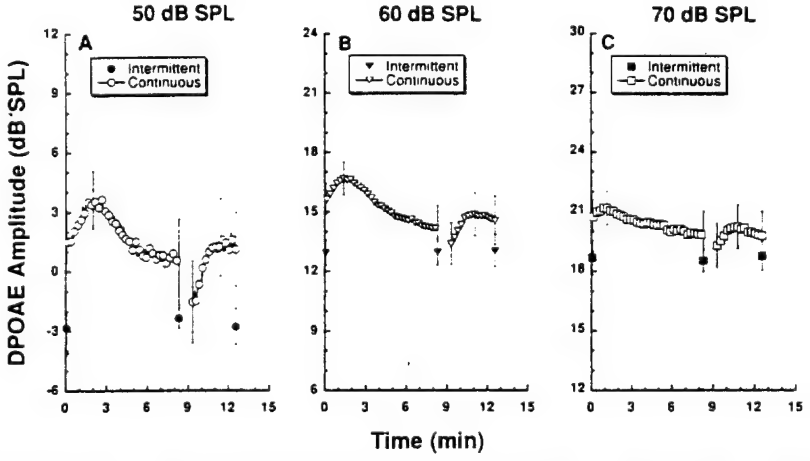

Fig. 4. Effect of primary intensity on f_2-f_1 DPOAE amplitude alterations ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 50–70$ dB SPL) associated with continuous (N = 9) and intermittent (N = 6) stimulations. Data are represented as means ± S.E.

Duration effects

In a subgroup of these animals ($N = 6$), DPOAE amplitudes to 50, 60 or 70 dB SPL primaries presented intermittently (at times corresponding to points A, C and F) remained very stable (Figs. 4A–C). On average, response amplitude to 60 dB SPL primaries varied less than 0.1 dB over a corresponding time period. We did not study how closely-spaced in time the periods of primary stimulation must be in order to produce these characteristic amplitude alterations. However, for periods of intermittent primary stimulation separated by 30 s intervals, distortion product recovery from the post-rest suppression followed a gradual course over the entire period of post-rest monitoring and the degree of amplitude recovery was more variable than that observed using the standard, continuous stimulation protocol.

Both the magnitude of the off-effect and the time course of the subsequent recovery were sensitive to the duration of rest from continuous stimulation. In general, the magnitude of the off-effect associated with either 30 s or 2 min periods of rest was < 0.5 dB. Moreover, although the times to maximum amplitude following the 1 and 2 min rests were similar, this time course was variable across animals following the abbreviated (30 s) rest.

Frequency effects

For these experiments, ($N = 5$) two additional frequency pairs were employed: $f_1 = 2.5$ kHz and $f_2 = 3$ kHz which yielded an f_2–f_1 DPOAE at 500 Hz and $f_1 = 10$ kHz and $f_2 = 12$ kHz which yielded an f_2–f_1 DPOAE at 2 kHz. The magnitudes of the amplitude alterations in the resulting f_2–f_1 DPOAEs were then compared to those observed with the standard primary pair ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz; f_2–$f_1 = 1.25$ kHz). No clear relationship to frequency emerged across the primary frequency range tested: In some animals, f_2–f_1 amplitude alterations were similar in magnitude for the 3 primary pairs tested and in some animals effects were larger when distortion products were elicited by mid- or high- but never low-frequency primaries.

DPOAE type

Under identical conditions of stimulation, amplitude alterations in the $2f_1$–f_2 DPOAE at 5 kHz were substantially smaller than those seen for the f_2–f_1 DPOAE at 1.25 kHz. In general, $2f_1$–f_2 amplitude increased slightly (< 1 dB) over the first few minutes of continuous primary stimulation and subsequently maintained this higher amplitude for the remainder of the stimulation period (Fig. 5). In none of these animals did we observe the continuous stimulation-related decline in $2f_1$–f_2 DPOAE amplitude observed for the f_2–f_1 DPOAE. In most animals, a small (approximately 0.5 dB), suppressive off-effect was evident. Our ability to observe amplitude alterations in this distortion product was not improved when stimulus intensity was reduced to 50 dB SPL, nor was it improved when $2f_1$–f_2 frequency was matched with that of the f_2–f_1 DPOAE under primary investigation in these experiments (1.25 kHz). In these latter groups of animals, however, on- and off-effects were occasionally reversed in sign – that is, on-effects appeared as small reductions in the amplitude of the $2f_1$–f_2 DPOAE and off-effects as small increases in response amplitude.

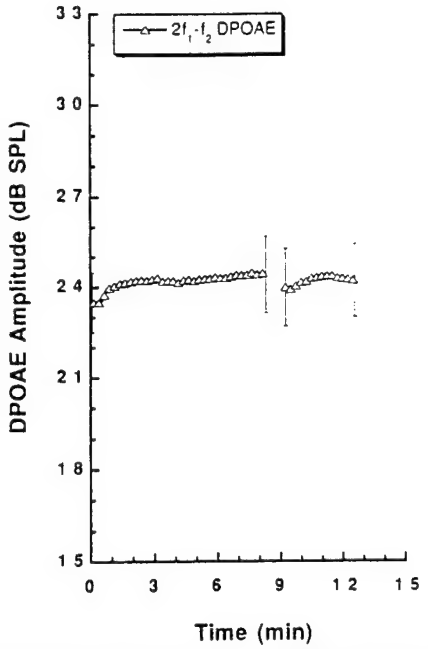

Fig. 5. Effect of continuous primary stimulation on $2f_1$–f_2 DPOAE amplitude ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). Data are represented as means ± S.E. across N = 4 animals.

3.4. Pharmacologic blockade of OC efferents

Control perfusions

In contrast to the remarkable stability of the $2f_1$–f_2 DPOAE to the manipulations associated with cochlear perfusion (e.g., Kujawa et al., 1993; Kujawa et al., 1994), the f_2–f_1 DPOAE was altered substantially by perfusion. All components of the perstimulus f_2–f_1 response alterations were enhanced by the first perfusion of the control solution (artificial perilymph; AP). In particular, the pre-rest amplitude suppression (ΔA–C) increased from an average of less than 3 dB pre-perfusion to roughly 8 dB post-AP #1. Similar enhancement of response suppression was not observed when primaries were presented only intermittently over the same time period, suggesting that the additional amplitude decline is not due solely to the long monitoring periods required following perfusion. Response alterations stabilized, however, following the initial AP perfusion and additional control perfusions ($N = 7$) in

each of 5 animals produced no further, significant changes in any response parameter. Thus, for each animal, values obtained following AP perfusion #2 were employed as the new post-perfusion baselines to which drug-related changes were compared. For all post-perfusion measures (control and experimental), contralateral suppression studies preceded immediately our monitoring of ipsilateral stimulation-related changes in f_2-f_1 amplitude. As a result of the prior stimulations, the initial on-effect (ΔA–B) described for the pre-perfusion measures is not observed in these post-perfusion amplitude records. The on-effect represented by the change in f_2-f_1 amplitude from points D–E remains, however, and was employed in statistical analyses of drug effects.

Drug perfusions

Intracochlear perfusion of bicuculline (10 μM) reduced, but did not block the component alterations in f_2-f_1 amplitude during ipsilateral stimulation (Fig. 6A). None of these drug-related changes reached significance ($P > 0.05$). Curare (Fig. 6B), at an order of magnitude lower concentration (1 μM), reduced significantly the pre-rest amplitude decline ($P < 0.05$), but drug-related changes in on- and off-effects failed to reach significance. In contrast, contralateral WBN effects on f_2-f_1 and $2f_1-f_2$ DPOAEs were blocked reversibly by both drugs (Figs. 7A–B), consistent with our previous findings for the $2f_1-f_2$ DPOAE (Kujawa et al., 1994). Also consistent with our previous findings, the amplitude of the $2f_1-f_2$ DPOAE, recorded in the absence of contralateral stimulation, was increased from levels observed following perfusions of artificial perilymph. We now extend those observations to include similar effects of these antagonists on f_2-f_1 DPOAE amplitude.

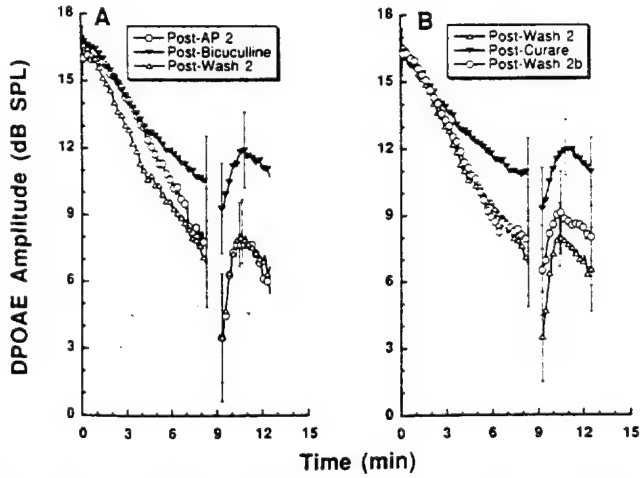

Fig. 6. Effect of OC efferent antagonists on f_2-f_1 DPOAE amplitude alterations during continuous primary stimulation ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). (A) Response amplitude means ± S.E.; N = 5) as recorded following the second control perfusion (AP2), bicuculline (10 μM) and the second wash perfusion W2). (B) Response amplitude (means ± S.E.; N = 5) as recorded following W2 above, curare (1 μM) and two additional wash perfusions (W2b). For reading ease, symbols occur only at every 3rd data point; connecting lines follow all data points.

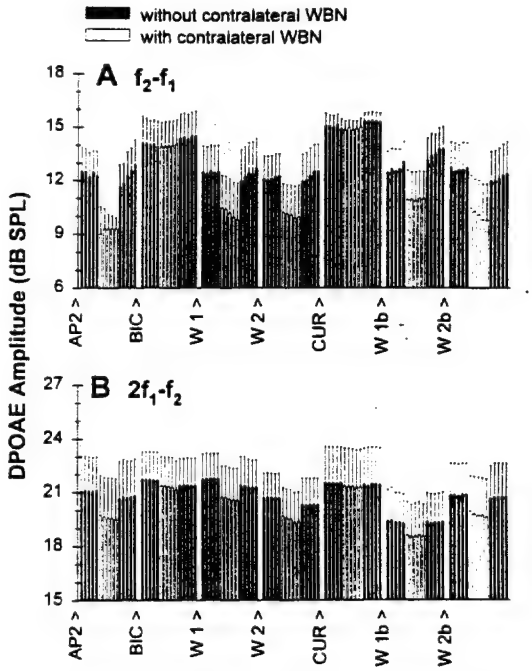

Fig. 7. Effect of OC efferent antagonists on contralateral WBN suppression of (A) f_2-f_1 and (B) $2f_1-f_2$ DPOAEs ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). In each panel, DPOAE amplitudes (means ± S.E., N = 5) are shown following perfusions of control solutions (AP2), bicuculline (10 μM, BIC), wash perfusions (W1, W2), curare (1 μM, CUR) and two additional washes (W1b, W2b).

In view of the long post-perfusion monitoring periods employed in these studies, it appeared possible that the inability of the drugs to block the ipsilateral effects might relate to a gradual washing of the drugs from the cochlear perilymph with replacement CSF before post-perfusion measurements could be completed. In pilot experiments, post-drug contralateral suppression was monitored after, rather than before ipsilateral stimulation measures and, in those animals, contralateral suppression could not be observed, suggesting that the drugs remained effective in blocking the efferents even at this extended time post-perfusion. Nevertheless, an additional subgroup of animals ($N = 4$) was tested in which the cochlear aqueduct was blocked prior to perfusion. In these animals, the magnitude of blockade of the ipsilateral effects was not altered substantially from that seen in aqueduct-patent animals. Of interest, when aqueduct-patent animals were subsequently anesthetized with Nembutal (30 mg/kg, i.p.) and ipsilateral stimulation measures repeated, reductions in the magnitudes of the component f_2-f_1 amplitude alterations were similar to those obtained following antagonist perfusions in urethane-anesthetized animals (see Fig. 8).

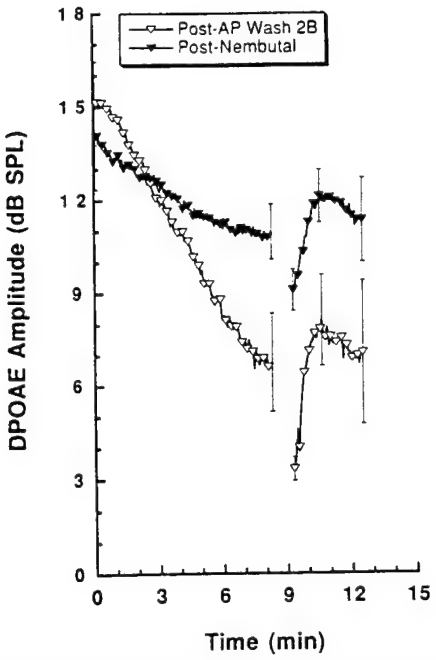

Fig. 8. Effect of Nembutal anesthesia on f_2-f_1 DPOAE amplitude alterations during continuous primary stimulation ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). Perfusion experiments completed under urethane anesthesia (AP Wash 2B) were followed by administration of Nembutal (30 mg/kg, i.p.) to N = 3 animals. Approximately 15 min later, post-Nembutal response measures were obtained. Symbols occur only at every 3rd data point; connecting lines follow all data points.

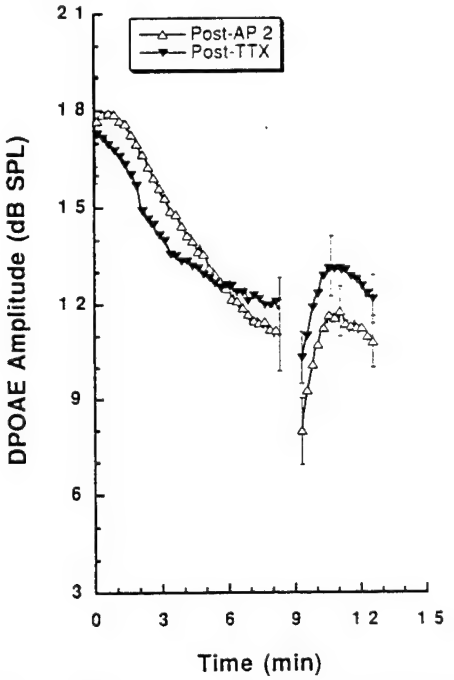

Fig. 9. Effect of TTX on f_2-f_1 DPOAE amplitude alteration during continuous primary stimulation ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kH $L_1 = L_2 = 60$ dB SPL). Shown are response amplitude means ± S.I (N = 6) following perfusions of control solutions (AP2) and TT (1 μM). Symbols occur only at every 3rd data point; connecting lin follow all data points.

The concentrations of OC antagonists employed in the perfusion experiments were chosen based on detailed studies of dose-response relations for the pharmacologic blockade of contralateral sound suppression (Kujawa et al., 1994). In those experiments, bicuculline (10 μM) and curare (1 μM) both were effective in blocking that efferent-mediated response. The substantially lesser potency of these drugs in blocking the ipsilateral effects under study here suggested that further clarification of the extent of efferent involvement was necessary. Thus, two additional manipulations were performed in separate groups of animals. In one group ($N = 6$), the cochlear perilymph was perfused with TTX (1 μM) which should block all action potential-mediated activity. Following such perfusions, the magnitude of the off-effect was reduced (Fig. 9), but the pre-rest amplitude decline did not differ significantly from baseline values ($P > 0.05$). In these same animals, contralateral suppression of both distortion products (Figs. 10A–B) was prevented ($P < 0.01$) and the round window-recorded auditory nerve compound action potential (CAP) at 10 kHz was not observed at any intensity (to 102 dB SPL) following perfusion of the cochlear perilymph with TTX. Figs. 10A-B also reveal that the absolute amplitudes of the f_2-f_1 and $2f_1-f_2$ DPOAEs were affected differently by this manipulation: The $2f_1-f_2$ DPOAE is reduced in amplitude

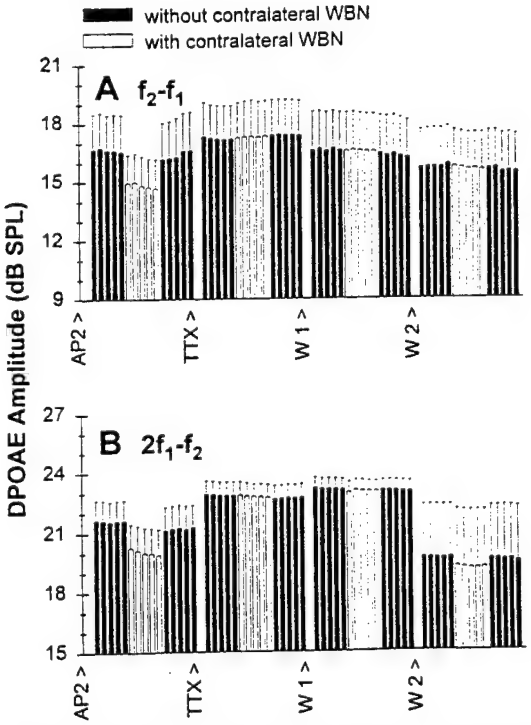

Fig. 10. Effect of TTX on contralateral WBN suppression of (. f_2-f_1 and (B) $2f_1-f_2$ DPOAEs ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, L_1 $L_2 = 60$ dB SPL). In each panel, DPOAE amplitudes (means ± S.I N = 6) are shown following perfusions of control solutions (AP2 TTX (1 μM) and two wash perfusions (W1, W2).

following attempts at washout; f_2–f_1 amplitude is little changed.

3.5. OC efferent section

A second manipulation performed to clarify the extent of OC involvement in these ipsilateral effects involved section of OC neurons at the midline of the floor of the IVth ventricle. The overall amplitude of the f_2–f_1 DPOAE was reduced following surgical exposure of the floor of the IVth ventricle, but the magnitudes of the component amplitude alterations in this distortion product were essentially unchanged (Fig. 11). Section of the OC efferents at the midline of the IVth ventricle floor produced no further changes in absolute DPOAE amplitude and did not alter substantially the f_2–f_1 amplitude alterations observed during continuous ipsilateral stimulation. In contrast, contralateral suppression of both f_2–f_1 and $2f_1$–f_2 DPOAEs was prevented by midline section (Figs. 12A-B). This may have occurred due to involvement of UMOC neurons in the midline cuts, as these fibers can course very near the brainstem midline, at least in cat (Gifford and Guinan, 1987) and mouse (Brown, 1993b). Here again, f_2–f_1 and $2f_1$–f_2 distortion components

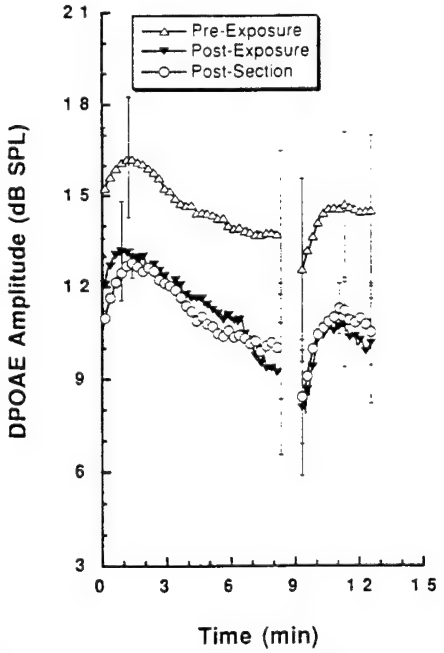

Fig. 11. Effect of OC section on f_2–f_1 DPOAE amplitude alterations during continuous primary stimulation (f_1 = 6.25 kHz, f_2 = 7.5 kHz, $L_1 = L_2$ = 60 dB SPL). Shown are response amplitude means ± S.E. (N = 4) prior to exposure of the IVth ventricle (Pre-Exposure), following IVth ventricle exposure (Post-Exposure) and following midline section of OC fibers (Post-Section). Symbols occur only at every 3rd data point; connecting lines follow all data points.

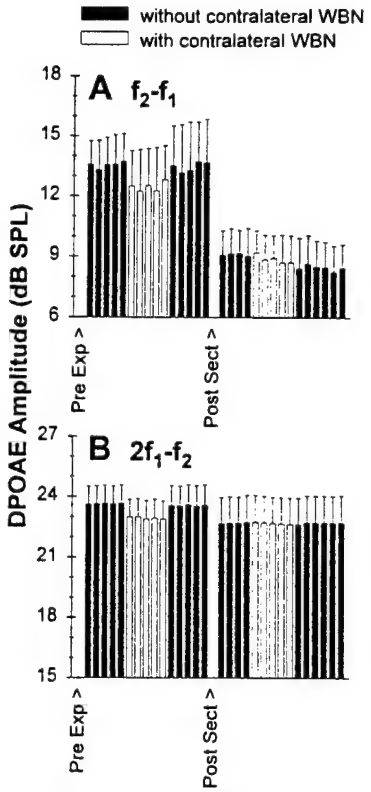

Fig. 12. Effect of OC section on contralateral WBN suppression of (A) f_2–f_1 and (B) $2f_1$–f_2 DPOAEs (f_1 = 6.25 kHz, f_2 = 7.5 kHz, $L_1 = L_2$ = 60 dB SPL). In each panel, DPOAE amplitudes (means ± S.E., N = 4) are shown before exposure of the IVth ventricle (Pre-Exp) and following midline OC section (Post Section).

display different post-manipulation behaviors: The reduction in absolute distortion product amplitude seen for the f_2–f_1 DPOAE following exposure of the IVth ventricle is not reflected in the $2f_1$–f_2 DPOAE.

4. Discussion

4.1. Alterations in magnitude of auditory system response

Alterations in the magnitude of auditory system response during and following continuous stimulation are well documented. Alterations with short (ms) time courses have been observed in responses of afferent neurons (e.g., Kiang et al., 1965; Young and Sachs, 1973a) and with longer (min) time courses in behavioral responses to sound (e.g., Moore, 1968; Noffsinger and Tillman, 1970; Young and Sachs, 1973b). Response enhancements at the onset of stimulation, perstimulatory declines and post-stimulatory depressions in response magnitude all have been observed. Of importance here, such alterations have been observed for stimulations at intensity levels that should not produce permanent, deleterious effects on cochlear structures.

4.2. f2–f1 amplitude alterations

Given these observations, it should not be surprising that time-varying alterations in the amplitude of the f_2–f_1 DPOAE are observed during continuous primary stimulation. The perstimulatory amplitude changes reported here are qualitatively similar to those reported by others for similar conditions of stimulation. Both Brown (1988) and Whitehead et al. (1991) reported initial increases in f_2–f_1 DPOAE amplitude that were followed by gradual declines during continued primary stimulation. Consistent with results presented by Whitehead and colleagues, we observed the response amplitude maximum (on-effect) to occur approximately 2 min into the period of continuous stimulation. Thereafter, response amplitude declined gradually over the remaining period of stimulation. Although the magnitude of this amplitude decline was similar across animals for 60 dB SPL primaries, it became substantially more variable as primary level was reduced. Brown (1988), employing lower primary levels (20–55 dB SPL), also reported large variations in magnitude of the f_2–f_1 amplitude decline. This variability may account, at least in part, for the reported differences in magnitude of effect between the various investigations of this phenomenon.

A suppressive off-effect of continuous primary stimulation has not been reported by previous investigators of these f_2–f_1 amplitude alterations. In reviewing those reports, it was noted that rest periods from primary stimulation employed in those studies generally exceeded the 1 min rest employed here. Furthermore, when the distortion product was monitored by Brown (1988) after a 3 min rest, partial recovery of response amplitude already was apparent. In the present experiments, the magnitude of this off-effect was substantially reduced when rest periods of either 30 s or 2 min were employed. Thus, it is not surprising that such additional response suppression was not observed by Whitehead et al. (1991) who employed rest periods of 5 min or longer and Brown (1988) who generally employed rest periods exceeding 2–3 min. According to the present results, the lengths of rest used by these investigators would likely have precluded their observation of this time-dependent effect.

4.3. 2f1–f2 amplitude alterations

Consistent with earlier investigations of these perstimulatory DPOAE amplitude changes, corresponding changes in the amplitude of the $2f_1$–f_2 DPOAE were very small or were not observed. In psychophysical paradigms, quadratic (f_2–f_1) and cubic ($2f_1$–f_2) nonlinearities can display different behaviors (Goldstein, 1967; Zwicker, 1979). Moreover, these two distortion components can exhibit different vulnerabilities to cochlear insult (Kujawa and Bobbin, unpublished observations; see also Figs. 10 and 12 in the present report). Such observations suggest that the mechanisms underlying generation of cubic and quadratic cochlear nonlinearities are not identical (for discussion, see Brown, 1993a).

4.4. Contralateral suppression

Although Kirk and Johnstone (1993) failed to observe convincing alterations in $2f_1$–f_2 amplitude during periods of contralateral WBN stimulation, results obtained in the present experiments are in excellent agreement with previous reports of $2f_1$–f_2 amplitude suppression by contralateral noise (Kujawa et al., 1993; Kujawa et al., 1994; Puel and Rebillard, 1990; Puria et al., 1992). Consistent with the report of Kirk and Johnstone, we also observed contralateral sound suppression of the f_2–f_1 DPOAE in virtually all animals tested. On average, the magnitude of this suppression was slightly greater than that observed for the $2f_1$–f_2 DPOAE although, in individual animals, it could be substantially greater.

4.5. Sites and mechanisms underlying response modulation

One possible mechanism that might account for the f_2–f_1 amplitude changes observed during continuous ipsilateral stimulation involves modulation of the cochlear mechanical response by OC neurons. This efferent influence would be accomplished via neurotransmitter (and neuromodulator) substances. Based on the finding of bicuculline (10 μM) blockade of ipsilateral and contralateral stimulation-related alterations in f_2–f_1 amplitude, Kirk and Johnstone (1993) suggested that both processes are controlled via GABAergic efferent pathways. In the present experiments, however, the GABA antagonist, bicuculline, at a concentration of 10 μM, was less effective than the nicotinic cholinergic antagonist, curare, at 1 μM, in antagonizing the perstimulatory amplitude reductions in f_2–f_1. Neither drug blocked these ipsilateral effects completely yet both drugs blocked completely contralateral sound suppression of f_2–f_1 and $2f_1$–f_2 DPOAEs. Our ability to pharmacologically antagonize these ipsilateral effects was not improved substantially in animals in which the cochlear aqueduct had been blocked prior to perfusion. Finally, neither TTX nor OC section altered substantially the ipsilateral stimulation-related effects.

We have previously characterized the pharmacology of contralateral sound suppression of the $2f_1$–f_2 DPOAE response to primaries within the range studied by Kirk and Johnstone (Kujawa et al., 1994). Results of those studies suggested that a nicotinic-like

cholinergic receptor mediated such suppression. Specifically, nicotinic antagonists (α- and κ-bungarotoxins, curare) and strychnine were most potent (IC_{50} values were achieved at nanomolar concentrations of these drugs). Bicuculline also was surprisingly effective in blocking this response suppression ($IC_{50} = 2.39 \times 10^{-6}$). Nicotinic receptor sensitivity to blockade by both strychnine and bicuculline has been demonstrated in several systems (see Kujawa et al., 1994 for review). This sensitivity has been suggested to reflect a strong structural homology between the receptors belonging to the super family that includes receptors for ACh, glycine and GABA (e.g., Grenningloh et al., 1987; Schofield et al., 1987).

At present, we cannot explain the differences between the two investigations with regard to the magnitudes of effect of bicuculline and TTX. The discussion of bicuculline's effects by Kirk and Johnstone (1993), however, suggests that substantial variability between animals in magnitude of drug effect was encountered. Perfusion also increased the variability in the f_2–f_1 DPOAE in our own experiments – both for control and experimental solutions. Additional procedural differences between the investigations may have contributed to observed differences in drug effects, as well. In the present experiments, middle ear muscles were sectioned; in the Kirk and Johnstone studies, animals were paralyzed in an attempt to prevent middle ear muscle contraction. There are differences in anesthetic agents employed and in placement of perfusion and effluent holes in the cochlea between the two investigations.

In view of the lack of effect of TTX and OC section on these response alterations, we also cannot explain the partial blockade of these amplitude alterations by the OC antagonists. However, Nembutal produced a blockade of the ipsilateral effects indistinguishable from that associated with bicuculline and curare. This effect certainly cannot be due to selective or specific blockade of receptors for the OC neurotransmitter. Rather, this finding suggests that, at these concentrations, bicuculline, curare and Nembutal may be acting nonselectively to block a channel operating in the OHCs. Evidence for channel blockade by bicuculline and curare was provided at the level of isolated OHCs by Erostegui et al. (1994).

4.6. Does efferent control underlie f_2–f_1 DPOAE amplitude alterations?

Results of the present experiments do not make it possible to specify the mechanism(s) underlying the perstimulatory alterations in f_2–f_1 amplitude described here. The results suggest, however, that they are not primarily the result of efferent control. Moreover, as reviewed by Young and Sachs (1973a), since sound-induced increases in efferent firing rate are maximum at stimulus onset (Fex, 1962) and OC inhibition of afferent activity decreases with time during a maintained stimulus (Wiederhold and Kiang, 1970), it follows that efferent activation should be associated with a perstimulatory response suppression which is maximum near stimulus onset, rather than the observed enhancement of suppression with time. The experiments cannot rule out an entirely peripheral efferent effect on f_2–f_1 DPOAE amplitude; however, if such an influence exists, it does not appear to involve action potential-mediated activity. Additionally, it would have to follow a time course much longer than that described to date for OC-mediated effects – even the recently-described 'slow effects' of OC stimulation (Sridhar et al., 1994) which demonstrated a pharmacology consistent with that previously described for contralateral suppression (Kujawa et al., 1994). A second mechanism that might underlie these ipsilateral effects could involve local, adaptive changes occurring at the level of the hair cells. Adaptation of the hair cell transduction current during sustained mechanical deflection has been demonstrated in vertebrate (although not yet mammalian) hair cells (e.g., Crawford et al., 1989; Eatock et al., 1987; Assad and Corey, 1992). Further, it has been suggested that distortion product responses arise from nonlinearities in hair cell channel gating mechanisms (Jaramillo et al., 1993). It is possible that such adaptive changes could be reflected in otoacoustic emissions. In previous work by Siegel et al. (1982) temporary reductions in the amplitudes of f_2–f_1 and $2f_1$–f_2 distortion components recorded at the level of single afferent units were observed following stimulations as low as 60 dB SPL. The authors noted that these alterations resembled those observed following higher-level, fatiguing stimulations. In subsequent papers, we will report the results of experiments in which we investigated the influence of local, calcium-dependent mechanisms and chronic, moderately-intense noise exposures on these amplitude alterations.

Acknowledgments

The authors are grateful to Ted Glattke for discussions and to Han Wen for programming assistance. This work was supported by NIH DC00722 and DC00007; DAMD 17-93-V-3013, Kam's Fund for Hearing Research and the Louisiana Lions Eye Foundation.

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Hearing Research 97 (1996) 153–164

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Time-varying alterations in the f_2-f_1 DPOAE response to continuous primary stimulation
II. Influence of local calcium-dependent mechanisms [1]

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Received 4 January 1996; revised 27 March 1996; accepted 3 April 1996

Abstract

The distortion product otoacoustic emission (DPOAE) corresponding to the frequency f_2-f_1 displays stereotyped, time-varying amplitude alterations during continuous primary tone stimulation. The origin of these alterations is unknown; however, evidence that efferent neurons contribute little to the changes has been presented (Kujawa et al., 1994a,1995; Lowe and Robertson, 1995). The present investigation examines the hypothesis that these alterations in f_2-f_1 amplitude are a reflection of local, Ca^{2+}-dependent mechanisms involving the outer hair cell (OHC) response to sustained stimulation. Experiments were performed using urethane-anesthetized guinea pigs with sectioned middle ear muscles. Intracochlear perfusion was employed to reversibly lower perilymph Ca^{2+} levels and to introduce antagonists and agonists of L-type Ca^{2+} channels. Manipulations that lowered available Ca^{2+} (zero Ca^{2+} artificial perilymph; zero Ca^{2+} with BAPTA) or that blocked its entry into the cell via L-type Ca^{2+} channels (nimodipine) reduced, prevented or reversed the perstimulatory changes in f_2-f_1 DPOAE amplitude. These perilymph manipulations also reduced the overall amplitude of this distortion component while perfusion of an L-type Ca^{2+} channel agonist (Bay K 8644) increased its amplitude. Mg^{2+} did not substitute for Ca^{2+}, suggesting that these are not merely divalent cation effects. Results are consistent with the hypothesis that continuous stimulation-related changes in f_2-f_1 DPOAE amplitude are sensitive to perilymph Ca^{2+} levels and to the function of L-type Ca^{2+} channels. However, nimodipine also reduced the endocochlear potential (EP) and Bay K 8644 increased the EP. The sensitivity of both the perstimulatory changes in f_2-f_1 DPOAE amplitude and the EP to the latter drugs leaves their site(s) of action unresolved.

Keywords: Adaptation; Calcium; Distortion product; Non-linearity; Outer hair cell; Otoacoustic emission

1. Introduction

The f_2-f_1 distortion product otoacoustic emission (DPOAE) displays stereotyped, time-varying amplitude alterations during continous, primary tone stimulation. If monitored after a period of silence, the f_2-f_1 DPOAE response to continuous primaries undergoes a short period of amplitude growth followed by a progressive reduction in amplitude during continued stimulation (Brown, 1988; Whitehead et al., 1991; Kirk and Johnstone, 1993; Kujawa et al., 1994a,1995; Lowe and Robertson, 1995). If the primaries are then removed and there is a 1 min rest from such stimulation, further response decrement is apparent immediately upon re-introduction of the primary tones (Kujawa et al., 1994a,1995). The response subsequently repeats its initial pattern of amplitude growth followed by slow decline as primary tone stimulation is continued. Similar amplitude alterations have not been observed in the cubic distortion product, $2f_1$-f_2.

The mechanisms underlying these stimulation-related changes in the f_2-f_1 DPOAE remain unclear. Medial olivocochlear (MOC) efferent neurons respond to sound (e.g., Liberman and Brown, 1986) and are anatomically well-situated to influence the response properties of outer hair cells (OHC). Electrical and acoustic activation of MOC neurons alters DPOAEs — effects presumed related to MOC influences on OHCs (Mountain, 1980; Puel and

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PII S0378-5955(96)00070-6

Rebillard, 1990). Thus, one possible mechanism that might account for the f_2-f_1 amplitude changes observed during continuous ipsilateral sound stimulation involves modulation of OHC activity by ipsilaterally responsive MOC neurons. However, two investigations that have tested this efferent-control hypothesis by studying the effects of antagonists of cochlear efferent activity (Kujawa et al., 1995) and of midline section of this fiber pathway (Kujawa et al., 1995; Lowe and Robertson, 1995) observed little impact of these manipulations on such changes in f_2-f_1 DPOAE amplitude. Thus, it appears unlikely that the efferent innervation to the cochlea plays a substantial role in producing these particular continuous stimulation-related f_2-f_1 DPOAE amplitude alterations.

A second mechanism that might underlie these effects involves local changes occurring at the level of the hair cells in response to sustained stimulation. Isolated OHCs are capable of responding to applied AC electrical stimulations with rapid, phasic changes in length (e.g., Ashmore, 1987; Brownell et al., 1985). If similar length changes occur in vivo in response to sound, they may allow the OHCs to amplify the mechanical stimulus to the inner hair cells (IHC) by providing a cycle-by-cycle boost at low stimulus levels (see Dallos, 1992 for review). Inherent in such length changes are non-linearities that are revealed as a tonic, or DC component that follows the envelope of the stimulus waveform (e.g., Santos-Sacchi, 1989; Evans et al., 1991). DC motions also can be observed in isolated OHCs in response to acoustic (mechanical) stimulations (Canlon et al., 1988; Brundin and Russell, 1994) and these motions are reflected in DC position shifts of the reticular lamina (Brundin et al., 1992) and basilar membrane (LePage, 1987). It has been suggested (Zenner and Ernst, 1993) that, in vivo, DC OHC length changes could influence the operating point of the stereocilia and, by changing their sensitivity to displacement, may contribute to such processes as adaptation during sustained stimulation and the temporary changes in sensitivity that follow overstimulation. Unlike the phasic (AC) component of the electromotile response, which is comparatively immune to trauma and does not require immediate metabolic (Ca^{2+}, ATP) support (Kachar et al., 1986; Ashmore, 1987), tonic (DC) motions of OHCs are highly vulnerable to overstimulation (Evans et al., 1991) and to pharmacologic manipulations and are metabolically dependent (Canlon and Brundin, 1991).

The mechanical response of the cochlear partition to sound, at least for lower levels of stimulation, is thought to reflect the physiologically vulnerable, active motion of the OHCs (Robles et al., 1991; Mammano and Ashmore, 1993). DPOAEs are a reflection of non-linearities in that mechanical response (Robles et al., 1991). Although the specific mechanisms underlying distortion product generation are not fully understood, they are widely regarded to directly involve the OHCs. With regard to the acoustic distortion product at f_2-f_1, Brown (1993, 1994) has argued that this non-linearity may be closely tied to the tonic motile response of OHCs to the beat cycle envelope of the two-tone stimulus. According to Brown, if these highly non-linear OHC length changes result in rectification of the response to bitonal stimulation, a difference frequency (f_2-f_1) would be generated which could propagate outward to be recorded as an acoustic distortion product emission. Such responses may provide an indirect means to study, in vivo, the OHC response to sustained stimulation.

We have speculated previously (Kujawa et al., 1994a,1995) that the slow changes in f_2-f_1 DPOAE amplitude that are observed during sustained, moderate-level primary stimulation are the result of Ca^{2+}-dependent, adaptive changes occurring at the level of the OHCs. The present investigation was undertaken to examine that hypothesis. Intracochlear perfusion techniques were employed to reversibly lower perilymph Ca^{2+} levels and to introduce antagonists and agonists of L-type Ca^{2+} channels. We studied the effects of these perilymph Ca^{2+} manipulations on the time-varying, perstimulatory changes in f_2-f_1 DPOAE amplitude. Preliminary results have been reported (Kujawa et al., 1994a).

2. Methods

2.1. Subjects

Experiments were performed on pigmented guinea pigs of either sex weighing between 250 and 400 g. Anesthetized animals (urethane, Sigma; 1.5 g/kg, i.p.) were tracheotomized and were allowed to breath unassisted. ECG and rectal temperature were monitored throughout each experiment and rectal temperature was maintained at 38° ± 1°C by a heating pad. Additional urethane was administered as required to maintain an adequate depth of anesthesia.

Surgical procedures have been described (Kujawa et al., 1994b). Briefly, cartilaginous ear canals were exposed and partially removed to allow direct and optimum coupling to the sound delivery system. In all animals the right auditory bulla was exposed using a ventrolateral approach and tendons of the right middle ear muscles were sectioned.

2.2. DPOAE: stimulus generation and response monitoring

DPOAEs (f_2-f_1, 1.25 kHz; $2f_1$-f_2, 5 kHz) were elicited by equilevel primary stimuli (f_1 = 6.25 kHz; f_2 = 7.5 kHz; f_2/f_1 = 1.2) delivered to the right ear of each animal by an acoustic probe assembly (see Kujawa et al., 1995 for additional details). Output from the probe microphone was led via a microphone preamplifier to a dynamic signal analyzer for fast Fourier transform analysis (averaging 10 discrete spectra) and spectral display (span = 1 kHz; CF = DP frequency; BW = 3.75 Hz). The noise floors associated with these display windows averaged approximately −12

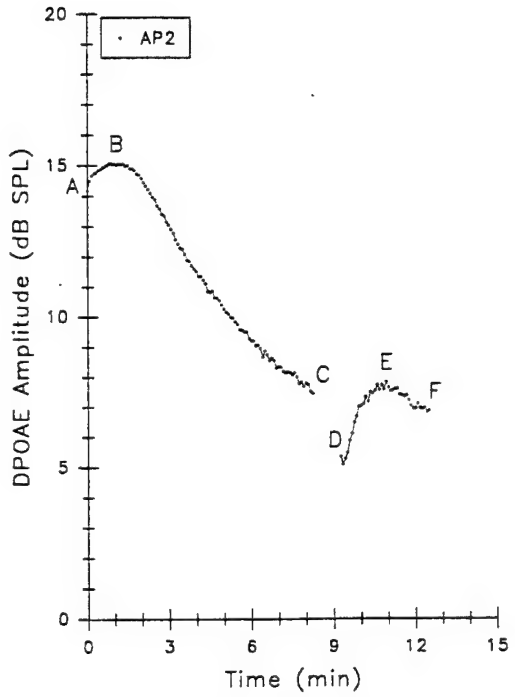

Fig. 1. A typical example of the effect of continuous primary stimulation on f_2-f_1 DPOAE amplitude (f_1 = 6.25 kHz, f_2 = 7.5 kHz, $L_1 = L_2$ = 60 dB SPL). Each data point represents a 10-spectra average and required 5 s to complete. Break in response amplitude trace (C–D) represents 1 min with no primary stimulation. Points A–F thus identified in each trace were used to calculate magnitudes of component amplitude changes for statistical analyses of treatment effects across animals. This particular trace was obtained following a control artificial perilymph perfusion (AP2).

dB SPL for the f_2-f_1 DPOAE and −15 dB SPL for the $2f_1$-f_2 DPOAE.

The f_2-f_1 DPOAE response to continuous, moderate-level (60 dB SPL) primaries was monitored as described in a previous report (Kujawa et al., 1995). In brief, 100 consecutive 10-spectra averages of distortion product amplitude were obtained during continuous primary stimulation. Each of these averages required approximately 5 s to complete for a total of 500 s (8.3 min) of stimulation. The primary tones were then simultaneously turned off and there was a 1 min rest from primary stimulation. Following this rest, the primaries were re-introduced and 40 consecutive 10-spectra averages of distortion product amplitude were obtained (total time ≈ 200 s or 2.3 min of stimulation). Six points (A–F) were identified on each response amplitude function (see Fig. 1). These values were used to characterize the component amplitude alterations as detailed elsewhere (Kujawa et al., 1995). They include an 'on-effect' (A–B), a 'slow decline' (B–C), an 'off-effect' (C–D) and a 'second on-effect' (D–E) following the return to stimulation. The distortion products at f_2-f_1 and $2f_1$-f_2 also were studied as growth functions of increasing primary level over the range 20–70 dB SPL in 5 dB steps.

2.3. Cochlear perfusion experiments

Perfusion studies were undertaken using methods described previously (Kujawa et al., 1994b). The 'normal' artificial perilymph had a composition of: 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM NaH_2PO_4, 1 mM $MgCl_2$, 11 mM glucose, and 12 mM $NaHCO_3$. An artificial perilymph without added Ca^{2+} ('0 Ca^{2+}' artificial perilymph) was obtained by omitting the $CaCl_2$ and the NaH_2PO_4. This solution also was used to dissolve and deliver the 5 mM BAPTA and the 2 and 4 mM Mg^{2+}. Nimodipine (Research Biochemicals International) and Bay K 8644 (S(−)-Bay K 8644, Research Biochemicals International) were dissolved in dimethylsulfoxide (DMSO, Sigma) and stored in the dark at −20°C for no more than 1 week. The nimodipine in DMSO, Bay K 8644 in DMSO, and DMSO alone were mixed with artificial perilymph on the day of use at desired concentrations (nimodipine: 0.1–10 μM; Bay K 8644: 0.1–10 μM; DMSO: 0.001–0.1%). Otherwise, all perfusion solutions were freshly prepared on the day of use. Perfusates (pH 7.4) were introduced at room temperature into the cochlear perilymph at a rate of 2.5 μl/min for 10 min (0 Ca^{2+}; 0 Ca^{2-} with 2 or 4 mM Mg^{2-}; 0 Ca^{2+} with BAPTA) or 15 min (DMSO; nimodipine in DMSO; Bay K 8644 in DMSO) through a hole in basal turn scala tympani and were allowed to flow from the cochlea through an effluent hole placed in basal turn scala vestibuli. Effluent was absorbed within the bulla using small cotton wicks. In all animals, the first two perfusions were of 'normal' artificial perilymph alone. These perfusions were accomplished to achieve a stable baseline to which effects of subsequent alterations in the artificial perilymph and drugs could be compared. They were followed by perfusions of the altered artificial perilymph ('0 Ca^{2-}' artificial perilymph) or experimental drug. Test solutions were washed from the cochlear perilymph with 'normal' artificial perilymph. A 10–15 min period without stimulation (during perfusions) separated each period of primary stimulation from the next. Following each period of perfusion, we monitored f_2-f_1 amplitude to 60 dB SPL primaries, presented continuously as described. Additionally, growth functions for this distortion component and the 5 kHz $2f_1$-f_2 DPOAE response to the same primary frequencies were obtained over the range 20–70 dB SPL in 5 dB steps. Data were obtained from five animals per treatment.

2.4. Endocochlear potential experiments

In separate groups of animals (n = 5 each), nimodipine, Bay K 8644 and the solvent DMSO were tested for their effects on the endocochlear potential (EP). Methods used to measure this potential have been described (Bobbin et al., 1990). Briefly, the bone over the basal turn scala media was shaved and a small hole was made through the thinned bone. A glass microelectrode, filled with 150 mM KCl and

connected to a DC amplifier (Grass P15), was passed through the hole and inserted into the scala media to record the EP. The output of the amplifier was connected to a digital voltmeter and chart recorder to obtain hard copy records of the EP values.

All perfusions in the EP study were 15 min in duration. The perfusion pump was turned on and the pipette was inserted into the infusion hole. Approximately 10 s later the first EP value was recorded. Additional measurements of the EP were obtained at 1 min intervals for 15 min after which the pipette was removed and filled with the next solution. Approximately 4 min elapsed between perfusions. The 16 values (0–15 min) were then averaged across animals to describe drug effects on the EP during the course of the perfusions.

Effects of treatments were quantified using repeated-measures analysis of variance (ANOVA) procedures and Newman-Keuls post-hoc tests. The care and use of the animals reported on in this study were approved by the university's Animal Care and Use Committee.

3. Results

3.1. Effects of continuous primary stimulation on the f_2-f_1 DPOAE

During continuous, equilevel primary stimulation (60 dB SPL), f_2-f_1 DPOAE amplitude displayed the time-varying changes we have described previously (Kujawa et al., 1995; see also Fig. 1). These amplitude changes include an initial increase in f_2-f_1 level that reached a maximum 1–2 min after the start of stimulation ('on-effect'; A–B), a 'slow-decline' in amplitude that followed with continued stimulation (B–C), the additional amplitude depression observed immediately following a short (1 min) rest from stimulation ('off-effect'; C–D) and the rapid recovery to a second maximum, again roughly 2 min following the return to continuous primary stimulation (second 'on-effect'; D–E). Such amplitude changes were observed during baseline response characterizations in all animals tested.

3.2. Actions of low Ca^{2+}, high Mg^{2+} and BAPTA on DPOAEs

Perfusion of the cochlea with an artificial perilymph solution without added Ca^{2+} (0 Ca^{2+}) had little effect on the component f_2-f_1 amplitude alterations to continuous primary stimulation (Fig. 2A). The most obvious effect was a small ($\approx$ 2 dB) reduction in the overall amplitude of the f_2-f_1 DPOAE response to 60 dB SPL primaries. When compared to values corresponding to points A, C and D (see Fig. 1 for reference) obtained following the second control perfusion (AP2), this overall amplitude reduction was not significant ($P > 0.05$). Subsequent perfusions of solutions containing no added Ca^{2+} with added Mg^{2+} (2 and 4 mM) resulted in larger amplitude reductions (Fig. 2B), with effects reaching significance ($P < 0.01$) for the 4 mM Mg^{2+} treatment. The magnitudes of the perstimulatory slow decline and 'off-effect' were significantly enhanced ($P < 0.01$) by both 2 and 4 mM Mg^{2+}, with the 4 mM Mg^{2+} solution actually reversing the direction of the 'off-effect'. In addition, the 'on-effect' maximum (point B) was shifted to progressively earlier points in time

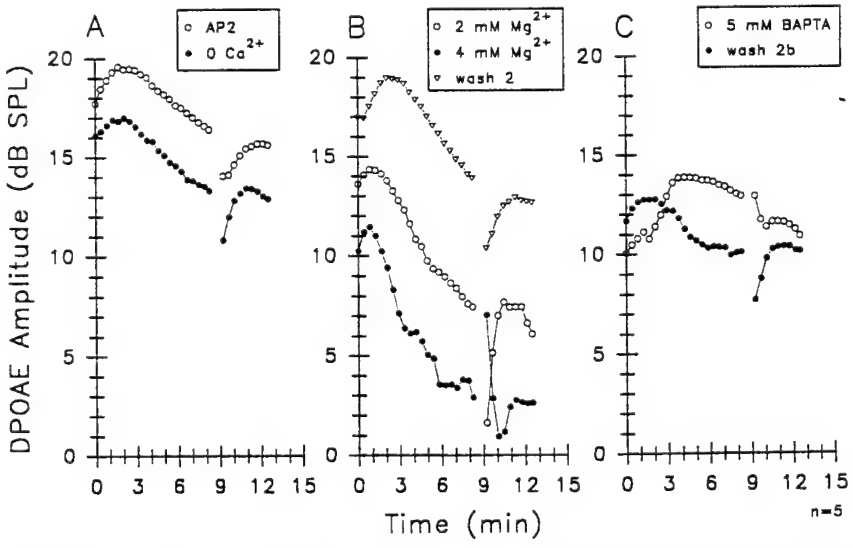

Fig. 2. Effect of altering perilymph Ca^{2+} or Mg^{2+} on perstimulatory changes in f_2-f_1 amplitude. Response amplitude (means: $n = 5$) recorded following successive perfusions in the same animals of: normal artificial perilymph (AP2), artificial perilymph with no added Ca^{2+} (0 Ca^{2+}), 0 Ca^{2+} artificial perilymph with 2 mM Mg^{2+} (2 mM Mg^{2+}) or with 4 mM Mg^{2+} (4 mM Mg^{2+}), a wash perfusion with normal artificial perilymph (wash 2), 0 Ca^{2+} artificial perilymph with 5 mM BAPTA (5 mM BAPTA) and a final wash with normal artificial perilymph (wash 2b). Only every 5th data point is plotted for clearer identification of symbol type. Pooled standard errors were: AP2 = 1.20; 0 Ca^{2+} = 0.75; 2 mM Mg^{2+} = 1.19; 4 mM Mg^{2+} = 2.55; wash 2 = 0.72; 5 mM BAPTA = 1.62 and wash 2b = 1.42 dB. See legend to Fig. 1 for additional information.

following perfusions of the 0 Ca^{2+} and the 0 Ca^{2+} with 2 and 4 mM Mg^{2+} solutions. All actions were readily reversed by perfusions with the 'normal' artifical perilymph (wash 2, Fig. 2B). The Ca^{2+} chelating agent, BAPTA (5 mM; in 0 Ca^{2+} artificial perilymph) was perfused next, and appeared to reverse the directions of all components of the perstimulatory f_2-f_1 DPOAE amplitude changes (Fig. 2C). These actions of BAPTA on the component amplitude alterations were reversed following wash perfusions with normal artificial perilymph (wash 2b) although overall distortion product amplitude did not return completely to pre-drug levels.

Drug-related changes in f_2-f_1 growth functions paralleled the effects observed for this distortion component during continuous primary stimulation (monitored in three of the five animals tested). Perfusion with artificial perilymph solutions without added Ca^{2+} (0 Ca^{2+}, 0 Ca^{2+} with Mg^{2+}) had little effect on low-level portions of the f_2-f_1 growth function (2 ± 4 dB at 40 dB SPL) and reduced f_2-f_1 amplitude at primary levels greater than ≈ 50 dB SPL (e.g., at 65 dB SPL, reductions of 3 ± 1 dB, 7 ± 2 dB and 9 ± 2 dB were obtained for 0 Ca^{2+}, 2 mM Mg^{2+} and 4 mM Mg^{2+} treatments, respectively). The $2f_1$-f_2 DPOAE also was monitored as a function of increasing stimulus level following drug perfusions. This distortion product was essentially unchanged by these perilymph manipulations (shifts averaged 2 dB or less at all stimulus levels). BAPTA in 0 Ca^{2+} artificial perilymph had large effects on both distortion components. Growth functions were shifted to the right, with greater effects seen at lower stimulus levels than at higher levels (e.g., the $2f_1$-f_2 DPOAE response to 40 dB SPL primaries was reduced by 8 ± 1 dB and the response to 65 dB SPL primaries was reduced by 4 ± 1 dB).

3.3. Actions of DMSO, nimodipine and Bay K 8644 on DPOAEs

Perfusion of the solvent (DMSO) alone, in the same increasing concentrations employed in the nimodipine and Bay K 8644 solutions, had little effect on the time-varying alterations in f_2-f_1 amplitude (Fig. 3). The post-DMSO decrease in the starting value of f_2-f_1 DPOAE amplitude (point A) was not significant ($P > 0.05$; Figs. 3 and 4), nor was the increase in the magnitude of the 'slow decline' (B–C). At higher concentrations, the 'off-effect' was progressively reduced; however, this reduction reached significance ($P < 0.05$) only at the highest DMSO concentration employed (0.1%).

In contrast to the small effects of the solvent alone, the

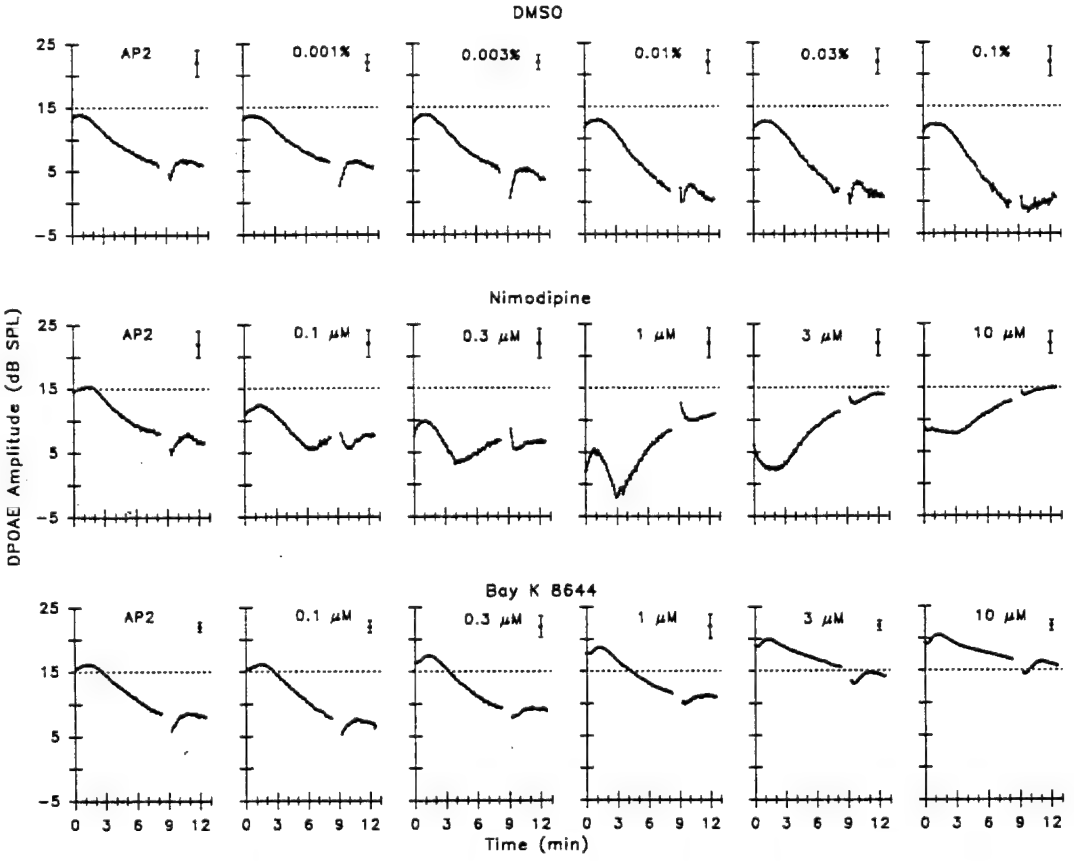

Fig. 3. Effect of increasing concentrations of DMSO, nimodipine in DMSO and Bay K 8644 in DMSO ($n = 5$ animals each) on the f_2-f_1 DPOAE response to continuous primaries at 60 dB SPL. Mean response amplitude for every data point is shown. Pooled errors are shown in the upper right hand corner of each frame. The dashed line is drawn at 15 dB for visual reference. See legend to Fig. 1 for additional information.

drugs had large effects. The actions of nimodipine were very complex (Figs. 3 and 4). The starting amplitude of the f_2-f_1 DPOAE (A) was decreased by 0.1–1.0 μM nimodipine. At higher concentrations (3 and 10 μM), this value began to return towards pre-drug levels. The 'on-effect' (A–B) was increased by the same low concentrations of nimodipine (0.1–1 μM), whereas higher concentrations (3–10 μM) appeared to reverse the 'on-effect'. It is possible that this reversal may have resulted from the slowly developing reversal of the 'slow decline' (B–C) which is first seen following perfusion of 0.1 μM nimodipine as an increase in f_2-f_1 magnitude approximately 6 min into the recording. With increasing drug concentrations, this amplitude recovery (or reversal of the slow decline) moves earlier in time: 0.1 μM = 6 min; 0.3 μM = 4 min; 1.0 μM = 3 min; 3.0 μM = 2 min and at 10 μM it may even begin at zero min. The 'off-effect' and the 'second on-effect' reversed at the lowest concentration of nimodipine tested (0.1 μM) and remained reversed over the course of the subsequent perfusions with increasing drug concentrations.

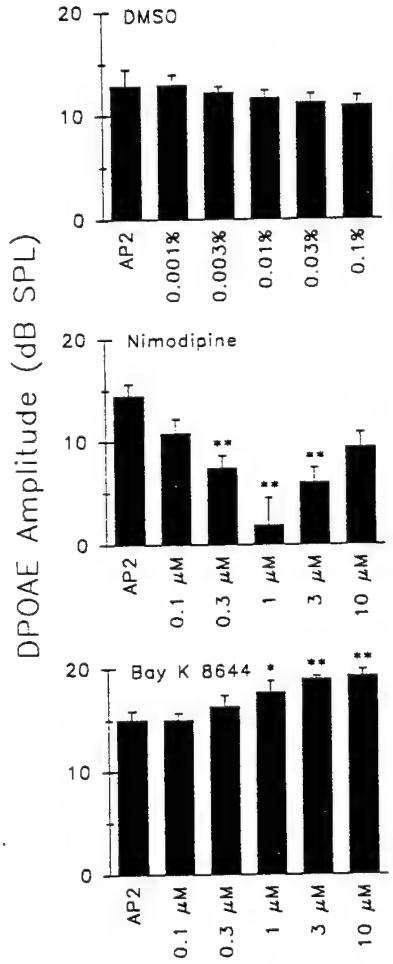

Fig. 4. Effect of increasing concentrations of DMSO, nimodipine in DMSO and Bay K 8644 in DMSO on the first f_2-f_1 DPOAE amplitude value recorded (point A) following each perfusion. Points that are significantly different from their respective AP2 or DMSO alone concentrations are designated: * $P < 0.05$; ** $P < 0.01$. Data are represented as means ± SE ($n = 5$ animals per drug treatment).

Compared to nimodipine, Bay K 8644 appeared to be very straightforward in its actions on the f_2-f_1 DPOAE (Figs. 3 and 4). Bay K 8644 increased, in a dose-related fashion, the absolute amplitude of this distortion component measured at point A. This increase reached significance ($P < 0.05$) for drug concentrations at 1–10 μM (Fig. 4). Bay K 8644 did not substantially alter the 'on-effects' (A–B; D–E) or the 'off-effect' (C–D). On the other hand, it did decrease the slope of the 'slow-decline' (B–C). Interestingly, the overall shapes of the response alterations after 3 and 10 μM nimodipine appear to be the reverse of the overall shapes observed after 3 and 10 μM Bay K 8644 (Fig. 3).

Drug-related changes in f_2-f_1 growth functions again paralleled effects observed during continuous primary stimulation (Figs. 5 and 6). DMSO had very little effect on the growth function, with the exception that it appeared to diminish the 'knee' in the function which occurred at approximately 60 dB SPL (Fig. 5). Nimodipine had complex effects in that low concentrations (0.1–0.3 μM) had little impact, a slightly higher concentration (1 μM) enhanced the function and higher concentrations (3–10 μM) reduced the low-to-moderate-level portions while continuing to enhance the higher-level portions of the growth functions. Bay K 8644 enhanced the growth function at all primary levels — especially for drug concentrations of 3 and 10 μM (Figs. 5 and 6). By comparison, neither DMSO nor Bay K 8644 altered $2f_1$-f_2 growth functions whereas nimodipine reduced them, especially at low levels of stimulation (Figs. 5 and 6).

3.4. Actions on the endocochlear potential

The first perfusion of artificial perilymph increased the EP slightly. The second perfusion of the artificial perilymph (AP2) did not alter the EP further. Thus, as with the DPOAEs, the AP2 value is considered the control for each series of perfusions. DMSO perfusions served as control for the concentration of DMSO in the drug solutions. For each experiment, the last (15 min) value of the EP recorded during the second control perfusion (AP2) was subtracted from the corresponding EP value recorded during perfusion of each concentration of drug. Dose–response curves of drug effects on the EP (means ± SE) were constructed from the resulting difference values and are displayed in Fig. 7.

Perfusions of increasing concentrations of the drug solvent, DMSO, had no significant effect ($P > 0.05$) on the EP when compared to its AP2 (Fig. 7). The experimental drug nimodipine produced a reduction of the EP that was significantly different ($P < 0.05$) from effects associated with its paired or equivalent DMSO concentration at all drug concentrations (0.1–10 μM). An estimated IC_{50} value for nimodipine of 0.42 μM was obtained using

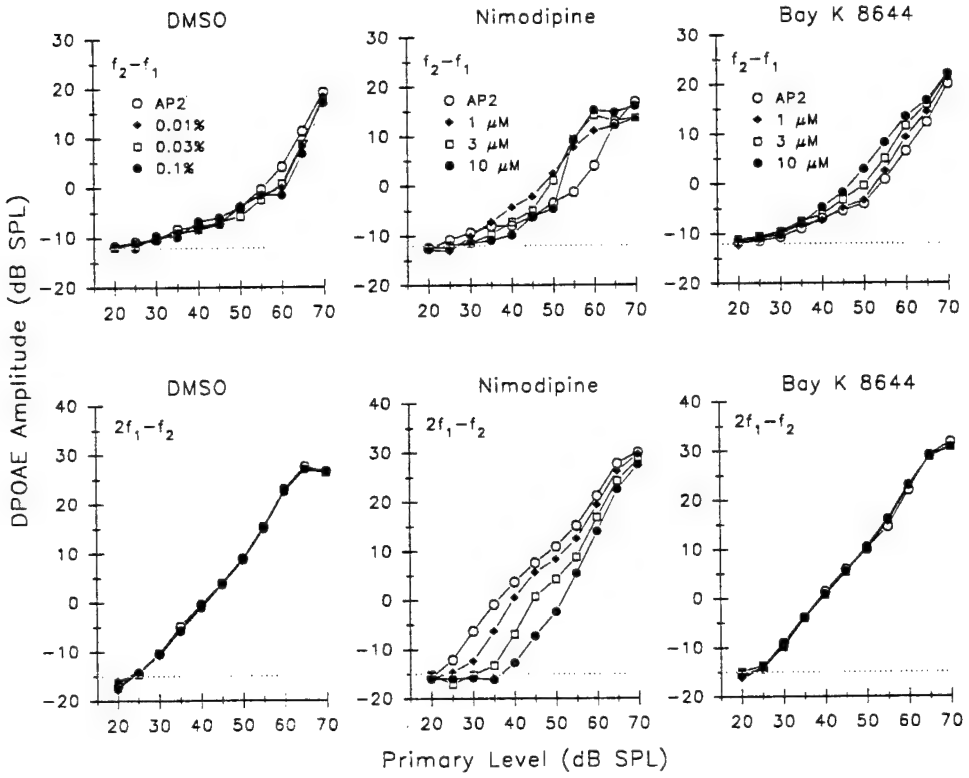

Fig. 5. Effect of perfusion of selected concentrations of DMSO, nimodipine in DMSO and Bay K 8644 in DMSO on growth functions for f_2-f_1 and $2f_1$-f_2 DPOAEs. Data are represented as means ($n = 4$ animals per drug treatment; see Fig. 4Fig. 6 for representative SE and statistics). The dashed line in each panel represents the average value of the noise floor.

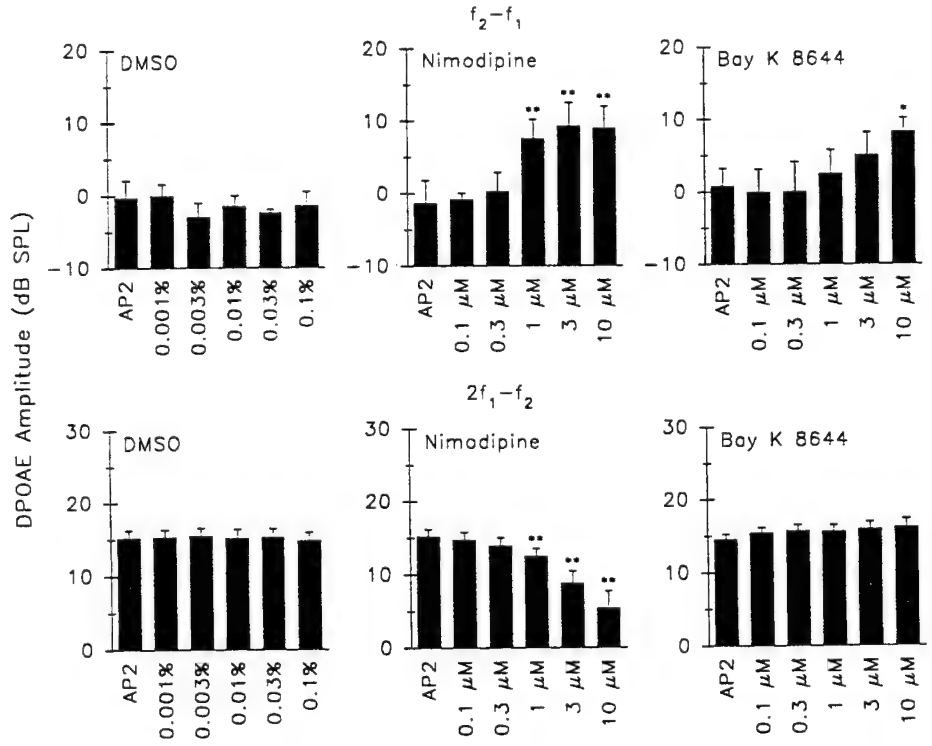

Fig. 6. Effect of perfusion of DMSO, nimodipine in DMSO and Bay K 8644 in DMSO on DPOAE amplitude to 55 dB SPL primaries. The values (means ± SE) are taken from those shown in Fig. 5 and subjected to statistical analysis. Values significantly different from corresponding AP2 are designated: * $P < 0.05$; ** $P < 0.01$ ($n = 5$ animals per drug treatment).

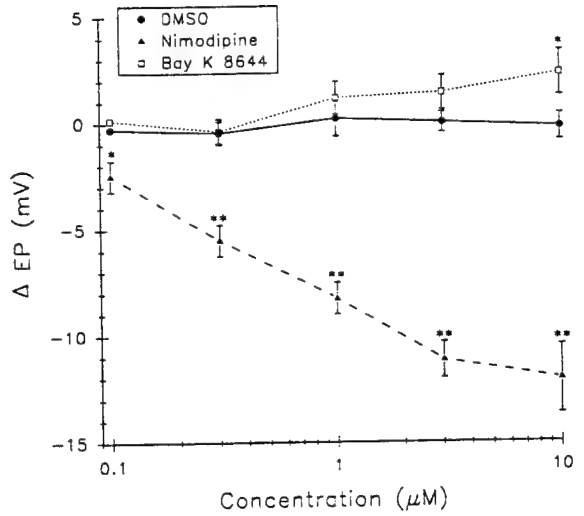

Fig. 7. Dose–response curves of the effects on the EP of DMSO, nimodipine in DMSO and Bay K 8644 in DMSO. Solvent (DMSO) concentrations corresponding to *X*-axis drug concentrations were as follows: 0.1 μM = 0.001%; 0.3 μM = 0.003%; 1.0 μM = 0.01%; 3.0 μM = 0.03%; 10 μM = 0.1%. For each experiment, the last (15 min) EP value obtained during perfusion of the second control perfusion (AP2) was subtracted from the last (15 min) EP value obtained during perfusion of each of the drug concentrations. The means ± SE of each of the values calculated in this manner are shown ($n = 5$ animals per drug treatment). Points significantly different from their respective DMSO-alone controls are designated: * $P < 0.05$; ** $P < 0.01$. Average drift in the EP records for each treatment was: DMSO = +3.4 mV; nimodipine = +0.6 mV; Bay K 8644 = +2.2 mV.

curve-fitting program (Origin®, 4.0). Bay K 8644 induced a small increase in EP that was significantly different only at 10 μM from its paired or equivalent DMSO concentration ($P < 0.05$; Fig. 7).

4. Discussion

4.1. General alterations in DPOAEs by the treatments

The present work demonstrates that perstimulatory changes in the amplitude of the f_2-f_1 DPOAE are altered by perilymph calcium manipulations. Manipulations that lowered available Ca^{2+} (0 Ca^{2-}; 0 Ca^{2+} with BAPTA) or that blocked the entry of Ca^{2+} into the cell through L-type Ca^{2+} channels (nimodipine) were associated with qualitatively similar alterations to the f_2-f_1 DPOAE. Specifically, overall amplitude of the f_2-f_1 DPOAE was reduced, as were the time-varying amplitude alterations during continuous stimulation. For the more potent manipulations (BAPTA; nimodipine), these amplitude changes ultimately reversed direction. Following perfusion of an L-type Ca^{2+} channel agonist (Bay K 8644) overall response amplitude was increased and the slope of the subsequent perstimulatory decline was reduced. Replacement of Ca^{2+} with Mg^{2+} did not reverse the effects, suggesting that these consequences of perilymph Ca^{2+} manipulation are not merely divalent cation effects. Growth functions for the f_2-f_1 DPOAE were altered more substantially than were growth functions for the distortion component at the frequency $2f_1$-f_2. Thus, in contrast to previous suggestions that the f_2-f_1 DPOAE is relatively insensitive to cochlear insult (see Brown, 1993 for review), we found it to be more vulnerable to the perilymph Ca^{2+} manipulations introduced in these experiments.

4.2. Sites and mechanisms underlying response to treatments

The cochlear mechanical response to low-level sound is thought to reflect the contribution of active and physiologically vulnerable motions of the OHCs (Robles et al., 1991; Mammano and Ashmore, 1993). DPOAEs are a reflection of non-linearities in that mechanical response (Robles et al., 1991) and may be the direct result of non-linearities in OHC length changes (Hu et al., 1994) or OHC channel gating mechanisms (Jaramillo et al., 1993). Thus, it seems reasonable to speculate that the alterations in f_2-f_1 amplitude observed during continuous primary stimulation are reflections of local, adaptive processes involving the OHC response to sustained stimulation and the effects observed subsequent to perilymph Ca^{2+} manipulations are related to alterations in these processes. There are several ways (either individually or in combination) that this could occur: (1) through alteration of a Ca^{2+}-dependent motility of the OHCs, (2) through an alteration to the Ca^{2+}-dependent adaptation of the OHC transduction current and (3) through an action of the drugs on the EP, secondarily altering OHC response characteristics.

4.2.1. Alterations to OHC motility

It is well known that isolated OHCs are capable of responding to applied AC electrical stimulations with 'fast' length changes that do not directly require Ca^{2+} or ATP (e.g., Ashmore, 1987). The tonic, DC length changes that can be observed during electrical and mechanical stimulations in vitro, however, appear to be much more vulnerable to insult (Evans et al., 1991) and dependent on metabolic support (Canlon and Brundin, 1991). Parallels between these non-linearities in OHC length changes and other non-linearities of the cochlear response (e.g., summating potential, SP; DPOAE) have been noted (Canlon and Brundin, 1991; Evans et al., 1991; Brown, 1993). In particular, at whatever level they are recorded, the non-linearities appear more sensitive to a variety of cochlear insults including pharmacologic manipulations and acoustic overstimulations (see Evans et al., 1991 for discussion). Of importance here, previous work from this laboratory (Bobbin et al., 1991) demonstrated a greater effect of perilymph Ca^{2+} manipulations on the SP than on the CM. In those experiments, lowering perilymph Ca^{2+} reduced the magnitude of the SP and increasing perilymph Ca^{2+} augmented the SP. In addition, the L-type Ca^{2+} channel

antagonist, nimodipine, reduced the magnitude and reversed the polarity of the SP (Bobbin et al., 1990). These are precisely the same effects observed on the non-linearity of the cochlear response under study in the present experiments — the f_2-f_1 DPOAE. An opposite effect, that of an increase in the magnitude of the f_2-f_1 DPOAE, was observed following perilymph perfusion of the L-type Ca^{2+} channel agonist, Bay K 8644. It is possible that all of these effects are secondary to Ca^{2+}-dependent changes in the non-linear motile response of OHCs and that these effects are mediated through an action at L-type Ca^{2+} channels.

An L-type Ca^{2+} channel has been described electrophysiologically and pharmacologically on OHCs of chick (Fuchs et al., 1990) and guinea pig (Nakagawa et al., 1991, 1992; Chen et al., 1995). These voltage-dependent Ca^{2+} channels are involved in a variety of cellular functions, including activation of contractile machinery in muscle cells and exocytotic secretion from endocrine cells and some neurons (see Kass, 1994 for review). L-type Ca^{2+} channels probably comprise the majority of the voltage-gated Ca^{2+} channels of guinea pig OHCs (Nakagawa et al., 1991; Chen et al., 1995).

It is generally assumed that the electromotile response of in vitro OHCs is driven by changes in membrane potential (e.g., Santos-Sacchi and Dilger, 1988). In vivo, under normal conditions of stimulation, it is likely that the OHC mechanomotile response to stereocilia deflection also will result from, or at least be strongly influenced by, changes in membrane potential (see Kolston, 1995). One difficulty with the hypothesis that L-type Ca^{2+} channels, in particular, are responsible for effects seen on the perstimulatory f_2-f_1 amplitude alterations is the level of depolarization required to activate the channels. Chen et al. (1995) reported that, in guinea pig OHCs studied in vitro, the L-type Ca^{2+} channel opened at roughly -30 mV. Because the normal resting membrane potential of in vivo OHCs is roughly -70 mV and sound-evoked depolarization of in vivo OHCs is usually reported to be less than 10 mV (e.g., Dallos and Cheatham, 1992), it is possible that the threshold of activation of the L-type Ca^{2+} channels would not be reached. However, Evans et al. (1991) note that, at least in vitro, the DC component of the motile response is sensitive to small depolarizations that do not necessarily alter the AC component. It is also possible that the activation curve reported for L-type Ca^{2+} channels in vitro is different in vivo.

It should be noted that nimodipine and Bay K 8644 can exert non-specific effects at ion channels other than L-type Ca^{2+} channels. Lin et al. (1995) recently reported that both of these agents can inhibit a late K^+ current in isolated OHCs maintained in Ca^{2+}-free media (0 Ca^{2+} with EGTA or BAPTA in external and internal solutions). In those studies, however, both nimodipine and Bay K 8644 produced the same effect (inhibition) which is not consistent with the effects of these drugs on the responses monitored in our experiments. Moreover, these effects were obtained at drug concentrations approaching the highest of those tested in the present experiments (IC_{50}s were 6 and 8 μM for nimodipine and Bay K 8644, respectively).

4.2.2. Alterations to Ca^{2+}-dependent adaptation of the OHC transduction current

An additional mechanism that could account for the results obtained is an alteration in processes related to adaptation of the OHC transducer channels. Transducer current adaptation during sustained stimulation is Ca^{2+}-dependent (e.g., Eatock et al., 1987). If the perstimulatory decline in f_2-f_1 DPOAE amplitude is related to these adaptive processes, reducing available Ca^{2+} should reduce the decline. This, along with an actual reversal of the decline, was the observed effect of perfusion with the Ca^{2+} chelator, BAPTA in 0 Ca^{2+} and with the L-channel antagonist, nimodipine.

According to Crawford et al. (1991), transducer current adaptation may be altered either by changes in the endolymph Ca^{2+} concentration or through changes in intracellular Ca^{2+} concentration. With regard to the former, Tanaka and Salt (1994) reported that cochlear function, as reflected in the EP, SP and CAP, was highly sensitive to small disturbances in Ca^{2+} concentration in endolymph. Ikeda et al. (1987) have suggested that, under normal conditions, the Ca^{2+} in endolymph is actively transported from perilymph. Thus, under conditions of reduced perilymph calcium, the concentration of Ca^{2+} at the stereocilia might be altered. Evans and Dallos (1993) have shown that conditions that interfere with mechanotransduction at the level of the stereocilia also interfere with the mechanomotile response of OHCs. Such responses of OHCs in a microchamber to stereocilia displacement disappear rapidly following exposure of the apical portions of the cells (cuticular plate and stereocilia) to an extracellular solution containing no added Ca^{2+} with 20 mM BAPTA. We consider it unlikely, however, that such alterations are responsible for the effects seen in the present experiments. Mechanotransduction can be maintained even with extremely low endolymph Ca^{2+} levels (Crawford et al., 1991) and, in vivo, there are a number of potential tissue pools for Ca^{2+} should such a reduction in endolymph Ca^{2+} occur. Moreover, nimodipine and Bay K 8644 should not affect overall Ca^{2+} levels in the endolymph or in other tissues, as the ion levels are controlled by transport and diffusion. Rather, the drugs likely act on proteins, possibly L-type Ca^{2+} channels.

Adaptation of the transduction current also can be affected at an intracellular site by Ca^{2+} that has entered the cell through a transducer or other membrane channel (possibly a voltage-gated Ca^{2+} channel in the basolateral membrane; Crawford et al., 1991). These investigators have speculated that during prolonged depolarizations, voltage-sensitive Ca^{2+} channels could be activated. Should the Ca^{2+} that enters via those channels gain access to the

transduction channels, adaptation would be facilitated. In this case, the effect on transducer current adaptation would not require an alteration in endolymph Ca^{2+}.

Finally, adaptation could be altered by processes that change the operating point of the stereocilia to mechanical stimulation. Zenner and Ernst (1993) have suggested that, by altering the position of the reticular lamina, DC OHC length changes could influence the operating point of the stereocilia. This, in turn, would modify their sensitivity, possibly altering transduction channel gating and contributing to such processes as adaptation during sustained stimulation and the temporary changes in sensitivity that are associated with overstimulation.

4.2.3. Alteration of the EP

The finding of drug effects on the EP complicates the interpretation of these results. Others have shown that distortion products recorded in the ear canal are sensitive to alterations in the magnitude of the EP (e.g., Mills et al., 1993). This sensitivity is related to the fact that the EP is necessary to power the OHC motile response (Dallos and Evans, 1995). In association with manipulations of the EP, Mills et al. (1993) observed a reduction or a reversal in the magnitude of the f_2-f_1 DPOAE similar to the effects we observed subsequent to perilymph Ca^{2+} manipulations. These investigators report that the magnitude of the $2f_1$-f_2 DPOAE is proportional to the square of the EP. We do not know whether the small changes in EP we observed following perfusion of Bay K 8644 (+2.3 mV) and nimodipine (−12 mV) are sufficient to account for the substantial changes in the responses we monitored, especially since the changes induced by Bay K 8644 are of approximately the same magnitude as the drift in the recordings (see legend, Fig. 7).

In most reports, merely altering perilymph Ca^{2+} levels does not induce changes in EP (Konishi and Kelsey, 1970; Sato, 1989; Bobbin et al., 1991). As noted previously, there are large potential pools for Ca^{2+} in the many fluid compartments of the cochlea, including blood vessels and cerebral spinal fluid entering via the cochlear aqueduct. Given this, it is not surprising that perfusion of low levels of Ca^{2+} through the perilymph compartment would have little impact on the function of the cochlea or on EP magnitude. Even the addition of Ca^{2+} chelators such as EGTA to the perfusate seems to have little effect on the EP (2 mM, Bobbin et al., 1991; 4 mM, Konishi and Kelsey, 1970; 4 and 10 mM, Sato, 1989). Perfusion of the Ca^{2+} chelator, BAPTA, nevertheless, had substantial effects on the continuous stimulation-related changes in f_2-f_1 amplitude recorded in the present experiments.

Nimodipine and Bay K 8644 also altered the perstimulatory amplitude changes in the f_2-f_1 DPOAE and both of these drugs had effects on the EP. Nimodipine and Bay K 8644 exert potent effects on L-type Ca^{2+} channel activity even in the presence of high levels of Ca^{2+} (Chen et al., 1995). Thus, they would not be affected by the large pools of Ca^{2+} in the tissue. Nimodipine significantly reduced the EP at the lowest concentration tested (0.1 μM), and effects increased with increasing drug concentration. At the highest concentration tested (10 μM), the EP was reduced by 12 ± 1.2 mV. This value is in good agreement with the 15 ± 3 mV reduction in EP associated with a single 30 min perfusion of 10 μM nimodipine reported previously (Bobbin et al., 1991). To our knowledge, there are no reports of the existence of L-type Ca^{2+} channels in the marginal cells of the stria vascularis, the structure generating the EP (e.g., Sunose et al., 1994). If these effects on the EP are due to drug actions at L-type Ca^{2+} channels, their location(s) remain to be determined. It is possible that L-type Ca^{2+} channels located at a site remote from the stria affected the EP. For instance, blockade of L-type Ca^{2+} channels in the OHCs by nimodipine may have induced the reduction in EP. Conversely, Bay K 8644 may have increased the EP by 'activating' an L-type Ca^{2+} channel on the OHCs (Chen et al., 1995).

5. Conclusions

Evidence that the amplitude alterations under study in the present experiments are not neurally mediated in general, and not efferent mediated in particular, has been reviewed (Kujawa et al., 1995). Briefly, these alterations in f_2-f_1 DPOAE amplitude were not prevented by the intracochlear application of TTX, which should block all action potential-mediated activity, and they were not prevented by antagonists of MOC efferent activity or by midline section of ipsilaterally responsive MOC neurons. However, efferent activity certainly can alter DPOAEs (e.g., Mountain, 1980; Siegel and Kim, 1982; Kujawa et al., 1993, 1994b). Of importance here, activation of ipsilaterally responsive MOC neurons by the primaries themselves has recently been reported to result in a rapid (time constant ≈ 100 ms) alteration in the amplitude of the $2f_1$-f_2 DPOAE (Liberman et al., 1996). Such response alterations disappeared immediately upon section of the crossed olivocochlear pathways and are clearly different from the very slow amplitude changes (time constant ≈ minutes) described in the present report.

In summary, previous studies from this laboratory (Bobbin et al., 1990; Bobbin et al., 1991) demonstrated that both lowering perilymph Ca^{2+} levels and perilymph application of the L-type Ca^{2+} channel antagonist, nimodipine, decrease the magnitude, and even reverse the sign of the DC receptor potential known as the SP. Results presented here extend this observation to DPOAEs, in particular the slow amplitude alterations in the f_2-f_1 DPOAE that are associated with sustained primary stimulation. We have speculated previously that such amplitude changes reflect local, Ca^{2+}-dependent, adaptive processes occurring at the level of the OHCs. The present results support that hypothesis and suggest, further, that the ef-

ts may be mediated, at least in part, by L-type Ca^{2+} nnels. Further study will be required to clarify the e(s) of action of nimodipine and Bay K 8644 in the chlea.

knowledgements

The authors wish to thank David Owens for programng assistance and Elaine McDonald and Amy Bayber technical help. This work was supported in part by search Grants DC00722 and DC00007 from the Nanal Institute on Deafness and Other Communication sorders, National Institutes of Health and DAMD 17-93-3013, Kam's Fund for Hearing Research and the uisiana Lions Eye Foundation.

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Appendix #4
DAMD17-93-V-3013
Bobbin and Berlin

In: Hair Cells and Hearing Aids. Edited by Charles H. Berlin. Singular Publishing Group, San Diego. 1996

2

Chemical Receptors on Outer Hair Cells and Their Molecular Mechanisms

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INTRODUCTION

We know that normal hearing depends on the proper functioning of active and passive cochlear mechanics and that the outer hair cells (OHCs) play a central role in the active mechanics. Various investigations have shown that the efferent nerves that innervate the OHCs influence cochlear mechanics by way of neurotransmitter chemicals released onto the OHCs. In addition, other cells in the organ of Corti and even the OHCs themselves may also release chemicals that act on the OHCs, that is, the chemicals act in a paracrine or autocrine manner. This chapter reviews the mechanisms of action of a few endogenous chemicals that act on OHCs with emphasis on the unique pharmacology of the efferent neurotransmitter, acetylcholine. The eventual goal of such studies is to understand the action of these molecules at the cellular and subcellular level and so understand the molecular mechanisms of cochlear mechanics.

Active and Passive Mechanics

The function of the cochlea is now thought of in terms of an active and passive mechanism as shown schematically in Figure 2–1. The passive mechanism is utilized during sound exposure over 40–60 dB SPL, where sound energy is powerful enough to move the cochlear partition directly.

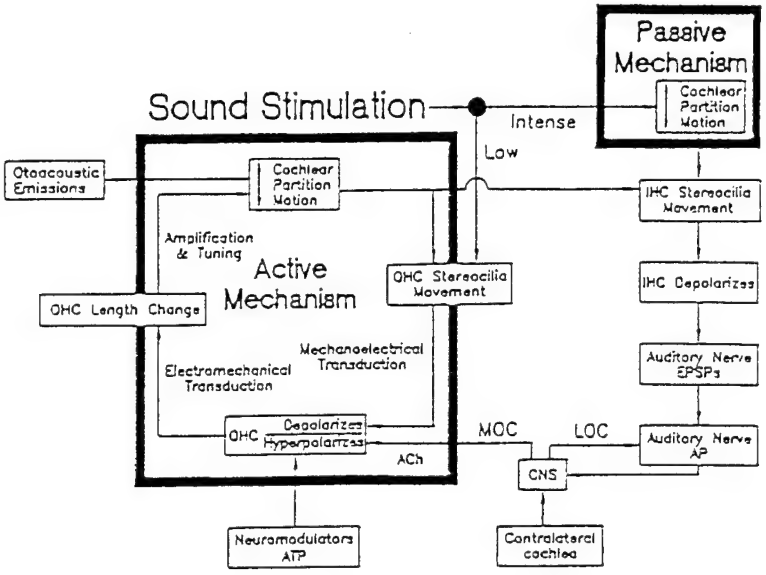


Figure 2–1. Schematic of the action of the passive and active mechanism for cochlear partition mechanics. IHC: inner hair cell; OHC: outer hair cell; LOC: lateral olivocochlear nerve tract; MOC: medial olivocochlear nerve tract; EPSPs: excitatory postsynaptic potentials; AP: action potential; ATP: adenosine triphosphate; ACh: acetylcholine; CNS: central nervous system. (Adapted from Pujol, 1990.)

This results in the movement of inner hair cell (IHC) stereocilia and opening of transduction channels which results in depolarization (less negative or more positive voltage) of the IHCs. Depolarization of the IHCs releases the neurotransmitter (i.e., glutamate) onto the auditory nerve endings which produces action potentials in the auditory nerve fibers. The active mechanism is utilized during low levels of sound exposure (<40 dB SPL) when the sound energy is insufficient to move the cochlear partition directly. Instead, in some unknown fashion, sound at this low level induces movement of the stereocilia on the OHCs. The movement of the OHC stereocilia opens transduction channels and depolarizes the OHCs (i.e., mechanoelectrical transduction). Depolarization of the OHCs changes the length of the OHCs (i.e., electromechanical transduction). This length change then results in amplification by inducing additional movement of the OHC stereocilia and greater depolarization and even greater length change of the OHC. The change in length of the OHC will move the cochlear partition and the greater the OHC length change, the greater the cochlear partition motion. When the movement of the partition is sufficient to induce IHC stereocilia movement, then events as described previously for the passive mechanism will take place, producing action potentials in

the auditory nerve. The amplification of the cochlear partition movement by the active mechanism contributes to the energy being transmitted back out through the ossicles in the form of otoacoustic emissions.

The OHCs are the key elements in the active process, with possibly some role for other cells such as Deiters cells (Dulon, 1994, Dulon, Blanchet, & Laffon, 1994; Dulon, Moataz, & Mollard, 1993; Moataz, Saito, & Dulon, 1992). Therefore, the OHC is a good anatomical point for the ear to control its response to sound. This is accomplished through several chemicals that act on the OHCs to alter their ability to change length in response to sound. As the motor of the OHCs is voltage controlled (Santos-Sacchi & Dilger, 1988), the degree of polarization of the OHCs determines the length of the OHCs: the more positive and farther away from its resting potential (@ –60 mV) the greater the shortening. Some chemicals depolarize the OHCs (i.e., membrane potential more positive) and this allows the OHC to shorten to a greater degree in response to sound. Others hyperpolarize the OHCs (i.e., membrane potential more negative) and this makes the OHC shorten less to sound. Other chemicals that affect intracellular chemical messengers are called second messengers (e.g., Ca^{2+}). Second messengers increase or decrease the length change in response to sound through a molecular mechanism inside the cell. The most studied chemicals are those released onto the OHCs from the efferent nerve fibers.

Efferent Innervation

Others have described the innervation of the cochlea by the olivocochlear efferent nerve fibers (e.g., Warr & Guinan, 1979). As shown in Figure 2–2, the medial portion of the olivocochlear efferents (MOC) synapse with the

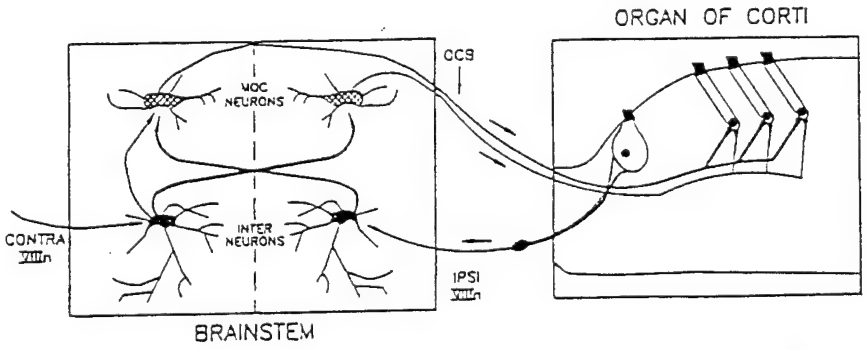


Figure 2–2. Innervation of the organ of Corti and the hair cells by the medial portion (MOC) of the olivocochlear nerve bundle (OCB) which originates in the brain stem. (Adapted from Liberman and T. Kawase, 1992.)

OHCs. Both contralateral and ipsilateral sound activate these MOC efferents and affect the activity of the OHCs by means of released chemical messengers. Thus, MOC nerve fibers play an important role in adjusting the active process. There are thought to be several chemicals involved in the mechanism of action of these efferent nerve fibers (see review by Eybalin, 1993). Here we will discuss only a few of the chemicals, with emphasis on acetylcholine (ACh).

NEUROTRANSMITTERS

Acetylcholine

Antagonists—in Vivo

There is no doubt that acetylcholine (ACh) functions as the primary neurotransmitter that the MOC neurons release onto the OHCs. This evidence comes from the pioneering studies by Guth, Norris, and others (see reviews by Bledsoe, Bobbin, & Puel, 1988; Daigneault, 1981; Eybalin, 1993; Guth, Norris, & Bobbin, 1976). ACh is a neurotransmitter at many locations, for example, the heart, neuromuscular junction. However, it appears that the receptor protein on the OHCs, to which ACh couples, is unique.

The uniqueness of the pharmacology of this ACh receptor protein was suggested in the earliest studies utilizing electrical stimulation of the MOC fibers in the brain stem and applying antagonists of various receptors intracochlearly to the perilymph compartment. Desmedt and Monaco (1960) were the first to demonstrate that strychnine applied to the round window (i.e., into perilymph) blocked the electrically induced action of these efferents (Figure 2–3). They suggested that the receptor protein was similar to the glycine receptor, since at that time strychnine was thought to be a specific blocker of neurotransmitter glycine. However, Churchill, Schuknecht, and Doran (1956) and Schuknecht, Churchill, and Doran (1959) had demonstrated that the efferent fibers stained for cholinesterase, suggesting that the efferent transmitter was ACh. Fex (1968) then demonstrated that curare, an antagonist of the ACh receptor at the neuromuscular junction, applied to the perilymph, blocked the efferents. This was some of the first evidence presented to show that the receptor protein on the OHCs was similar to the ACh receptor protein at the neuromuscular junction (Figure 2–4; because nicotine activates the ACh receptor at the neuromuscular junction, the receptor is called a nicotinic receptor—abbreviated Nm).

Table 2–1 is a partial summary of the pharmacology of this synapse obtained utilizing intracochlear perilymph application of the drugs and

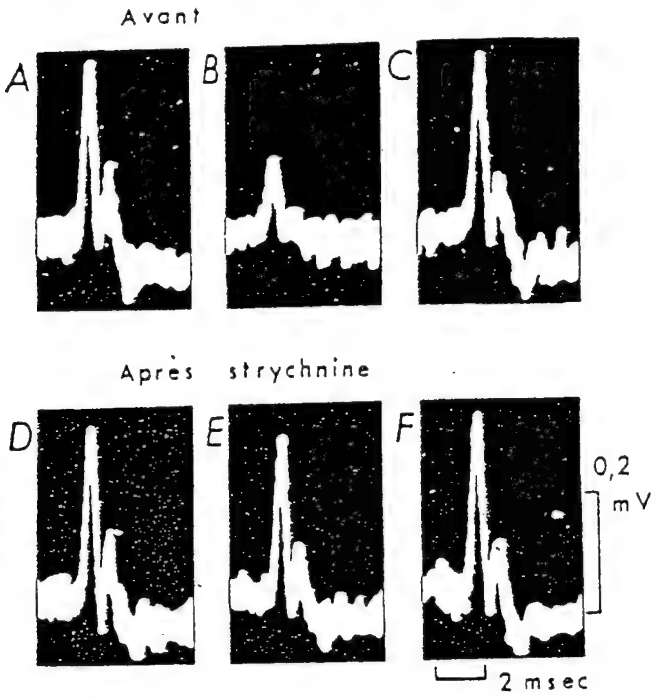


Figure 2–3. Oscilloscope traces showing the responses of the acoustic nerve (round window) stimulated by a click of 20 dB above threshold. Four successive traces are superimposed on each graph in order to show the stability of the response. In B and E, a train of 30 shocks (30 microseconds, 6 volts) at 300/sec was applied to the bundle of Rasmussen at the level of its decussation 20 msec before the tested click. The olivocochlear inhibition of the click response appears clearly when you compare the traces B and E to the responses of a single click (not preceded by the train) obtained before (A and D) and after (C and F) the stimulation of the bundle of Rasmussen. The administration of strychnine considerably reduced the olivocochlear centrifugal inhibition, but does not affect the response to the single click. (From Desmedt and Monaco, 1960, with permission.) (Translated by author)

electrical stimulation of the MOC efferents in the brain stem (from Bobbin & Konishi, 1971a, 1971b, 1974; Fex & Adams, 1978; & Galley, Klinke, Oertel, Pause, & Storch, 1973). For instance, Konishi and I found that the effects of the efferents could be blocked by atropine, a muscarinic receptor antagonist (the receptor found at the autonomic innervation of glands and smooth muscle and called a muscarinic type of receptor as it is activated by the drug muscarine, abbreviated M). Atropine exhibited less potency than either strychnine or curare. At the time, this unusual block by atropine was tempered by the fact that the quaternary atropine was

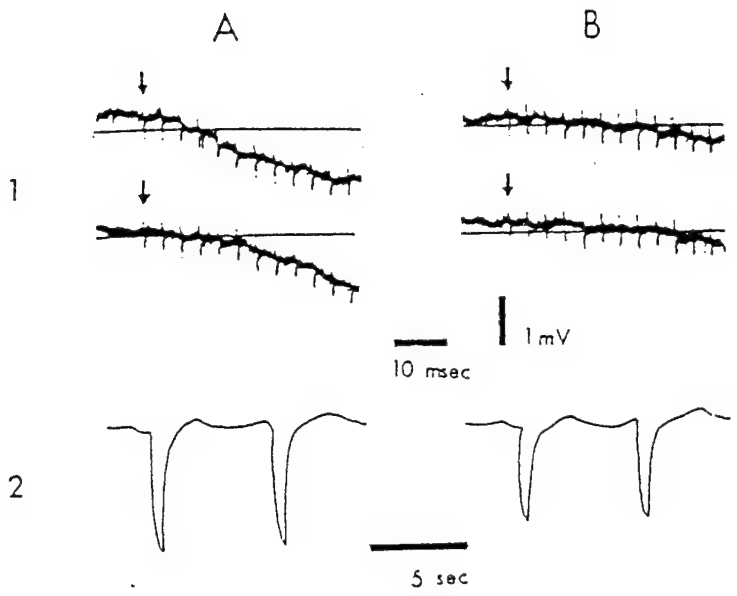


Figure 2–4. Records illustrating the action of d-tubocurarine on centrifugally evoked intracochlear potentials. Row 1 shows the initial time-course of potentials that were evoked by repetitive electrical stimulation of crossed olivo-cochlear fibres. Row 2 shows the full time-course of the potentials of Row 1. All the records were taken with the microelectrode in one position in the scala media. The start of stimulation is indicated by an arrow in Row 1. A. Control, artificial perilymph in the scala tympani. B. 12.5 minutes later than A. Artificial perilymph containing 1.0 μmole (0.7×10^{-6} g/ml) d-tubocurarine had, 5–8.5 minutes later than A, partly replaced the solution without d-tubocurarine. Note that after the application of d-tubocurarine, the time of rise and the latency of the potentials were prolonged and the amplitude decreased.

Note that voltage calibration is common to all the records, while time calibration is different for the two rows. The ink-writer that produced the records of Row 2 had a rise time constant of 0.15 seconds; d.c. recordings were used for all records. Negativity is downward. (From Fex, 1968, with permission.)

more potent than the tertiary atropine, a finding in harmony with a nicotinic type of receptor. In addition, we found decamethonium more potent than hexamethonium, suggesting that the receptor was like the acetylcholine receptor at the neuromuscular junction (nicotinic receptor, Nm) and less like the acetylcholine receptor at autonomic ganglia (nicotinic, Nn). Others reported that alpha-bungarotoxin, an antagonist at the Nm

TABLE 2–1. A summary listing of antagonists of the effects of electrical stimulation of the MOC on cochlear potentials. Also given is a list of the receptor types and the locations where these antagonists are most selective.

Receptor type	Location	Antagonist of MOC
Nicotinic (Nm)	skeletal muscle	curare, α-BTX, quaternary atropine, decamethonium
Nicotinic (Nn)	autonomic ganglia	hexamethonium
Muscarinic (M)	smooth muscles, glands	atropine
Glycinergic (gly)	central nervous system	strychnine

receptor, also blocked the efferents (Fex & Adams, 1978). Overall, it appeared that the receptor protein on the OHCs was similar to the Nm receptor, yet it was different because it was blocked by so many drugs that acted at other receptors. But because the experimental preparations were so complex, one was not sure where the drugs were acting. For instance, the action of strychnine was attributed to the drug blocking the release of ACh and not due to blocking the receptor (Fex, 1968, p. 183).

An additional problem with studying this efferent/OHC synapse using electrical stimulation of the MOC nerves was the difficult surgery and the instability of the preparation during the experiment. The discovery by Puel and Rebillard (1990) of a method of activating the efferents by sound to the contralateral ear yielded a technique that made the study of the pharmacology much easier. They monitored the effect of the efferents on the distortion product emissions (DPOAEs) which reflect the activity of the OHCs, and demonstrated that DPOAEs were suppressed by contralateral sound (Figure 2–5).

Kujawa, Glattke, Fallon, and Bobbin (1993) duplicated the Puel and Rebillard study and found that the response to contralateral noise was blocked by intracochlearly applied strychnine, curare, and atropine, with atropine being the least potent (Figure 2–6). In addition, the preparation was sufficiently stable so that Kujawa, Glattke, Fallon, & Bobbin (1994a) could generate cumulative dose response curves for the various types of pharmacological agents (Figure 2–7). Utilizing this technique, Kujawa et al. (1994a) demonstrated that the ACh receptor on the OHCs did indeed

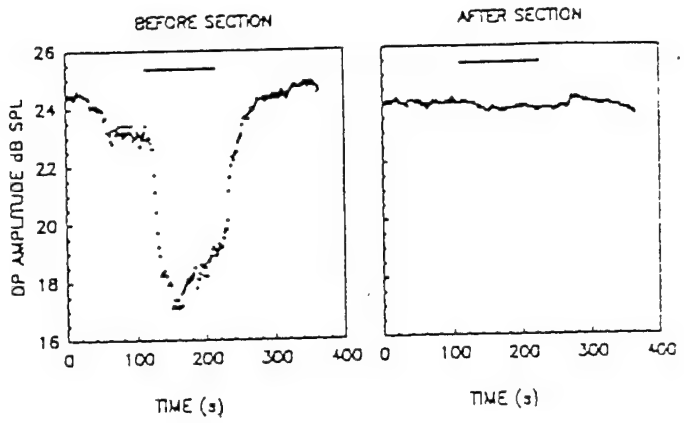


Figure 2–5. Effect of a midline sagittal section of the brainstem on 2F1–F2 DPs recorded at 5 kHz. The **left panel** represents the 2F1–F2 DPs reduction induced by a 100 dB SPL contralateral white noise. The **right panel** shows that after a complete midline sagittal section of the brainstem, the suppressive effect of the contralateral white noise is no longer active. The horizontal bars represent the time during which the contralateral white noise was presented. (From Puel and Rebillard, 1990, with permission.)

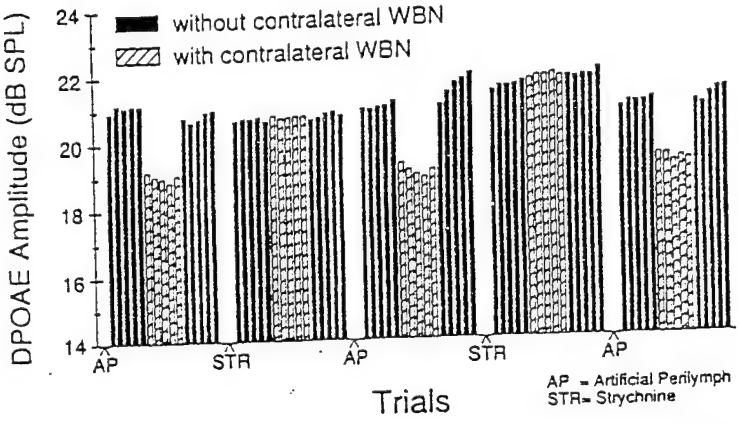


Figure 2–6. Effect of intracochlear strychnine on contralateral suppression of DPOAEs in one representative animal. DPOAE amplitudes are shown after the second control perfusion (artificial perilymph; AP) and after perfusions of 10 μM strychnine (STR) which were followed by artificial perilymph (AP) perfusions that washed out the drug. Each perfusion was 10 minutes in duration; post-perfusion measures are separated by an approximately 25 minute interval during which time the perfusion was completed, post-perfusion measures were taken, and the perfusion pipette was prepared for the next perfusion. Each "trial" represents a 50 spectra average and required 20 seconds to complete. Each set of post-perfusion measures thus required a total of approximately 300 seconds. Graph represents a total time of approximately 2.5 hours. (From Kujawa et al., 1993, with permission.)

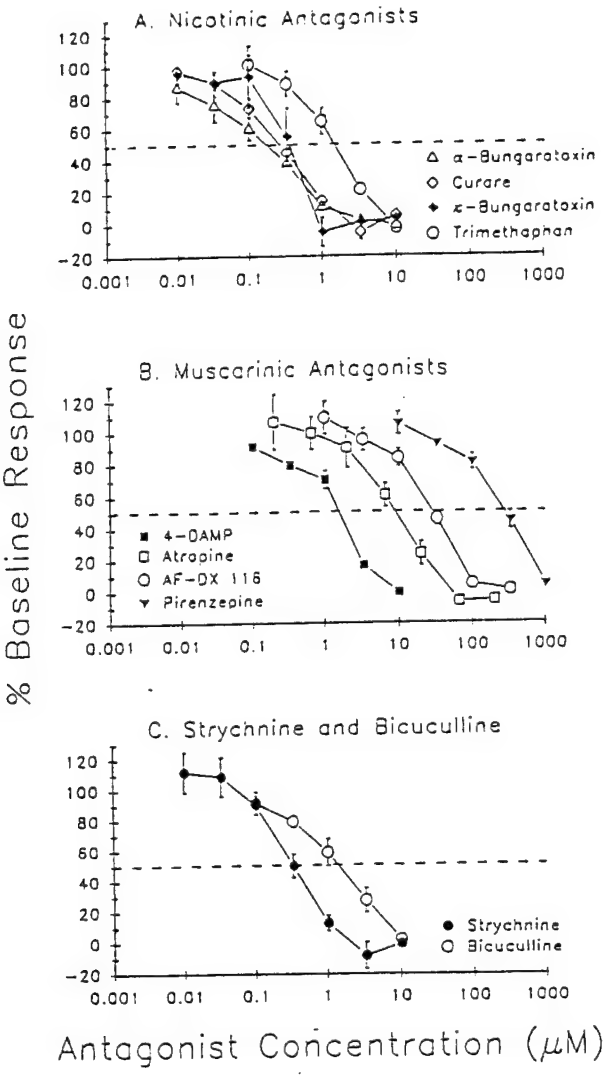


Figure 2–7. Inhibition curves (means ± S.E.) for antagonist blockade of baseline contralateral suppression. Curves associated with nicotinic antagonists are displayed in **panel A**, those associated with the muscarinic antagonists in **panel B** and the curves associated with the nontraditional cholinergic antagonists strychnine and bicuculline are shown in **panel C**. Magnitude of contralateral suppression following the second control perfusion established the baseline response for each animal. Magnitude of suppression as recorded following each concentration of experimental drug was then expressed as a percentage of this baseline response. Horizontal line in each panel designates the point of 50% blockade of baseline suppression (IC_{50}). For all drugs but κ-bungarotoxin, each set of means is based on $N = 5$ animals; for κ-bungarotoxin, $N = 2$ animals. (From Kujawa et al., 1994a, with permission.)

appear to have an unusual pharmacology: it was readily blocked by strychnine and by ACh antagonists. The drugs active at the neuromuscular junction (nicotinic, Nm, e.g., curare) were more potent than those active at autonomic ganglia (nicotinic, Nn, e.g., trimethaphan) or at smooth muscle (muscarinic, M, e.g., atropine). Kujawa et al. added another unusual property of the receptor: a block by the GABA receptor antagonist, bicuculline. Again because of the complexity of the preparation, each drug's exact site of action remains obscure, but the unusual pharmacology obtained with electrical stimulation was confirmed utilizing natural stimulation of the MOC nerve fibers.

Antagonists—in Vitro

Housley and Ashmore (1991) were the first to demonstrate that the ACh receptor protein's spectrum of pharmacology to ACh antagonists at the level of the isolated OHC was similar to that obtained using the whole animal (Figure 2–8; potency in blocking applied ACH: curare > strychnine > atropine > pirenzepine). Fuchs and Murrow (1992b) demonstrated a similar spectrum of pharmacological activity in the chick hair cells (Figure 2–9; potency: strychnine > timethaphan > curare > atropine). Kakeha-

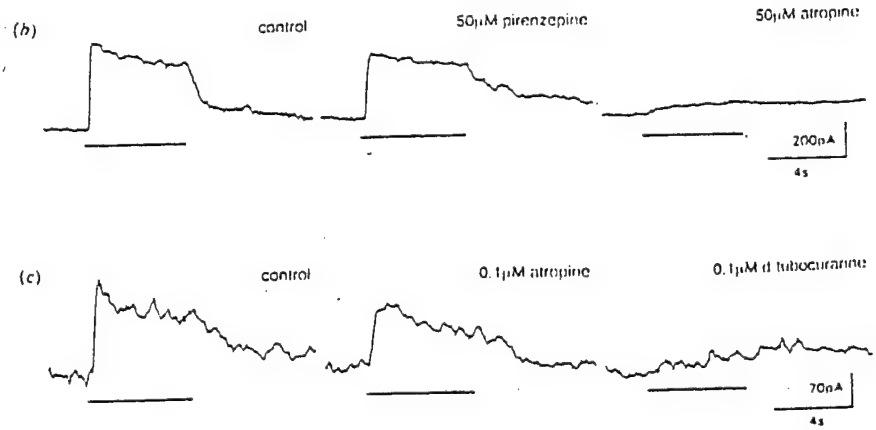


Figure 2–8. Localization and synaptic properties of the ACh response. (a) Not shown. (b) Atropine was more effective than pirenzipine as an antagonist when included at equimolar concentrations with 50 μM ACh. (c) The nicotinic antagonist d-tubocurarine was more effective than atropine when both were applied at 100 nM along with 50 μM ACh; 5 s pressure pulse monitored below. Holding potential, –50 mV in (a) -60 mV in (b, c). (From Housley and Ashmore, 1991, with permission.)

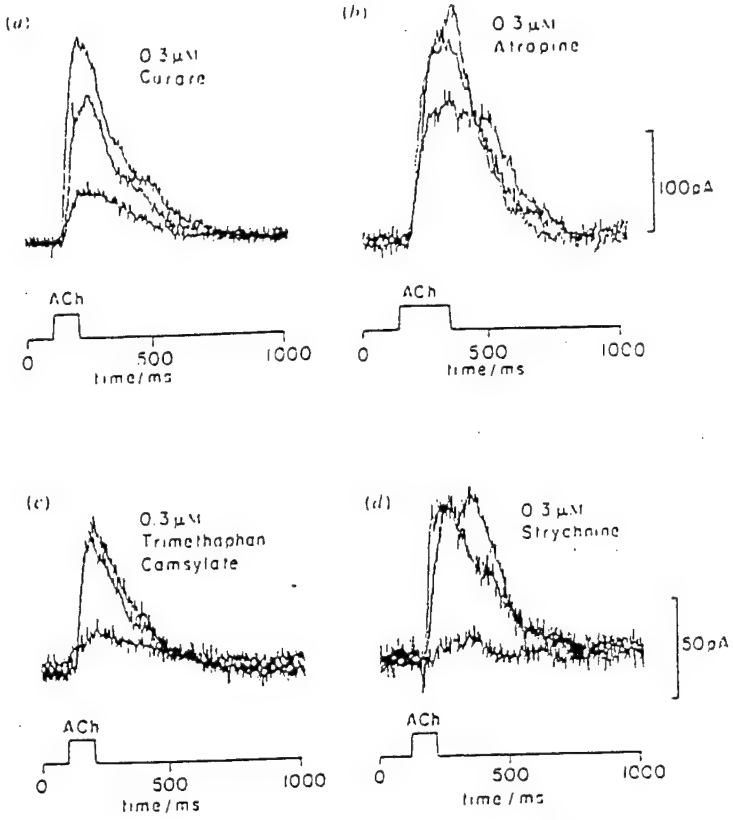


Figure 2–9. Antagonists of the hair cell ACh response. (a) Curare (0.3 μM) blocked 62% of the ACh (100 μM) response. Membrane potential –44 mV. (b) Atropine (0.3 μM) blocked 37% of the ACh response. Membrane potential –24 mV. (c) Trimethaphan camsylate (0.3 μM) blocked 77% of the ACh response. Membrane potential –54 mV. (d) Strychnine (0.3 μM) blocked 79% of the response. Membrane potential –54 mV. Application of ACh indicated by the lower bar. Pre- and post-controls shown in each panel. All antagonists were shown to be completely reversible in these or other cells. (From Fuchs and Murrow, 1992b, with permission.)

ta, Nakagawa, Takasaka, and Akaike (1993) tested muscarinic antagonists and found the following order of potency against applied ACh: atropine = 4-DAMP > AFDX 116 > pirenzepine. Erostegui, Norris, and Bobbin (1994) compared many of the drugs in the same preparation, extending the previous findings and showing that bicuculline blocks the effect of applied ACh (Figure 2–10; Table 2–2). Overall, the data of Erostegui et al. indicate the following order of potency of antagonists against applied ACh: strychnine, a glycine receptor antagonist > curare, a

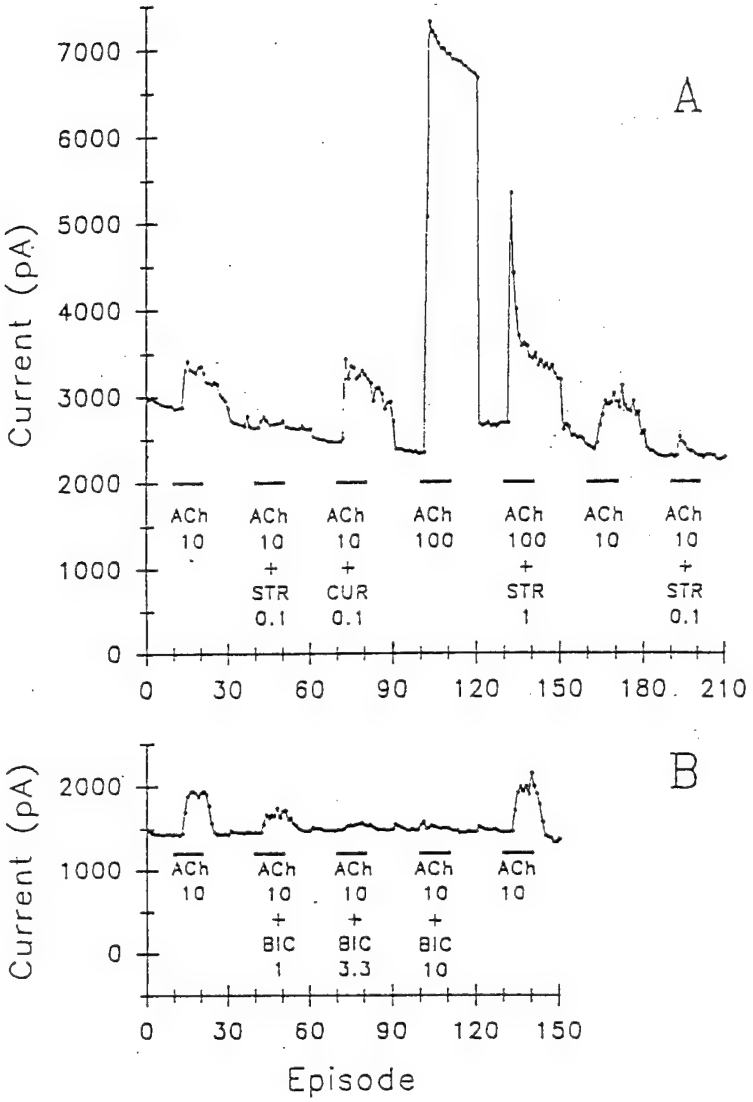


Figure 2–10. Effect of the nontraditional cholinergic antagonists, strychnine (STR) and bicuculline (BIC) on the response to ACh. A: strychnine (0.1 μM) blocked ACh. The large response (4.7 nA) to ACh (100 μM) is suppressed in great extent by strychnine at 1 μM. Bicuculline (3.3 and 10 μM) blocked ACh. Numbers near the drug abbreviations are drug concentrations in μM. Shown are data obtained as voltage clamp current responses at zero mV utilizing the same step protocol as illustrated and described in Figure 2–2. See Figure 2–2 for further description. (From Erosteguí et al., 1994, with permission.)

TABLE 2–2. Summary of effects of ACh antagonists on the ACh evoked current response in isolated OHCs.

	Concentration (in millimolar):								
ACh Antagonist	0.1	0.3	1	3.3	10	33	100	330	1000
Curare	0%	–	87%	100%					
Trimethaphan Camsylate	0%	–	40%	100%	100%				
α-bungarotoxin	0%	0%	50%	100%					
Atropine	–	–	0%	45%	100%	100%			
4-DAMP	–	–	0%	25%	66%	100%			
AFDX	–	–	–	0%	44%	100%			
Pirenzepine	–	–	–	–	–	–	50%	100%	100%
Strychnine	75%	–	100%						
Bicuculline	–	–	75%	90%	100%				

Source: Erostegui et al. (1994).

Note: Shown are the concentrations of antagonist (millimolar) tested against the current induced by 10 μM ACh in single isolated OHCs. Percentage indicates the number of cells in which the induced current was blocked out of the number of cells tested with the concentration of antagonist.

Nm receptor antagonist > bicuculline, a GABA receptor antagonist > atropine, a M receptor antagonist. Surprisingly, the pharmacological data obtained in vivo matched fairly closely with the data obtained in vitro. Because these studies used single cells and studied the blockade of applied ACh, the drugs were all acting at the OHC membrane, probably at the ACh receptor protein. However, even single cell experiments are complex; some of the drugs that blocked the action of applied ACh may have been acting at ion channels or other sites on the OHC and not directly on the OHC receptor protein. More studies will have to be done to prove the site of action.

Agonists–in Vivo

The use of antagonists characterizes a receptor protein to a certain extent. On the other hand, agonists can give more information, as these are the chemicals that activate the receptor protein to initiate subsequent cellular events. For instance, when ACh was combined with eserine (which blocked cholinesterase from degrading the ACh molecule) and placed into the cochlear fluids, it not only mimicked the effects of efferent stimulation, but in the face of the continuous application of ACh the response declined or desensitized while concurrently the efferents became ineffec-

tive (Bobbin & Konishi, 1971a, 1971b; Figure 2–11). Kujawa, Glattke, Fallon, and Bobbin (1992) replicated this earlier work by utilizing DPOAEs and again demonstrated desensitization. These studies demonstrated that ACh could act at this synapse in a manner similar to activation of the MOC efferent fibers. In addition, they demonstrated desensitization of the response and emphasized the activity of cholinesterase in destroying applied acetylcholine. More importantly for this discussion, Konishi and I demonstrated that neither nicotine, a powerful agonist at the nicotinic receptors, nor arecoline, an agonist at muscarinic receptors, had much of an effect (Bobbin & Konishi, 1971a, 1974). Galley et al. (1973) described

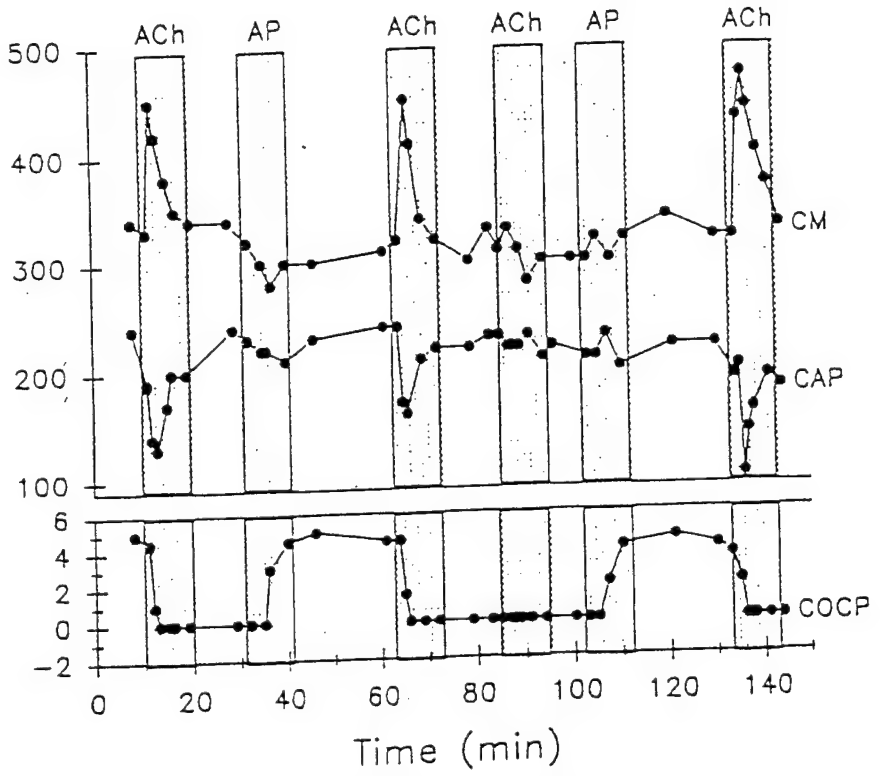


Figure 2–11. Effect of perfusion of the scala tympani on the cochlear microphonic (CM) (600 Hz, 64 dB SPL) and auditory nerve potential (AP) (6 kHz, 81 dB SPL) recorded without COCB stimulation, and the slow potential change (COCP) elicited by COCB stimulation. The scala tympani was perfused with artificial perilymph (A) and with acetylcholine chloride (250 μM) together with eserine sulphate (10 μM; B). (From Bobbin and Konishi, 1971b, with permission.)

the low potency of the muscarinic agonist, muscarone. Thus, even though the data with antagonists demonstrated the receptor was nicotinic (i.e., Nn), the data with agonists suggested the receptor was not nicotinic or muscarinic as the agonists had no effect.

Agonists–in Vitro

Recently, Kakehata et al. (1993) confirmed the in vivo data utilizing individual isolated OHCs. They demonstrated very little or no activity with nicotine, muscarine, McN-A-343, oxotremorine, and oxotremorine-M (Figure 2–12). They found that the most potent agonists were acetylcholine and carbachol. Erostegui et al. (1994) found that not only were nicotine and muscarine ineffective, but so was cytisine (Figure 2–13). On the other hand, Erostegui et al. found that DMPP and suberyldicholine, additional nicotinic agonists, were active.

Table 2–3 summarizes the unique pharmacology of this unusual receptor. The ACh receptor protein appears to have an unusual spectrum of activity in response to the various antagonists; the antagonists listed in Table 2–1 all block the ACh receptor on OHCs even though they are specific for the other receptors listed (e.g., pirenzepine vs the M1 receptor). In addition, the receptor fails to be activated by either the M receptor agonist, muscarine, or the Nn and Nm receptor agonist, nicotine. Cytisine, an additional nicotinic receptor agonist also failed to activate the receptor. On the other hand, it was activated by DMPP and suberyldicholine. To the best of our knowledge, no receptor to date has been described with this spectrum of pharmacological activity (e.g., Seguela, Wadiche, Dineley-Miller, Dani, & Patrick, 1993), although recent results with an alpha 9 receptor subunit come very close (Elgoyhen, Johnson, Boulter, Vetter, & Heinemann, 1994). So it appears that the receptor protein on the OHCs may be unique and not described to date.

In previous publications, I argued that the receptor was nicotinic, but this was based on the pharmacology to the antagonists. On the other hand, it is difficult to name it nicotinic when nicotine has no effect. In general, receptors are named after the drug most active at the receptor, for example, nicotinic or muscarinic. So it seems then that the ACh receptor on the OHC should not be called nicotinic or muscarinic. As suberyldicholine is one of the most potent agonists, the receptor should be called a suberyldicholinic receptor, or "subdic" for short (Bobbin, 1994).

Receptors have been grouped into families based on their molecular configuration. For instance, at present ACh receptors are classified as belonging either to the nicotinic family or the muscarinic family. At present we do not know to which family the ACh receptor on the OHC

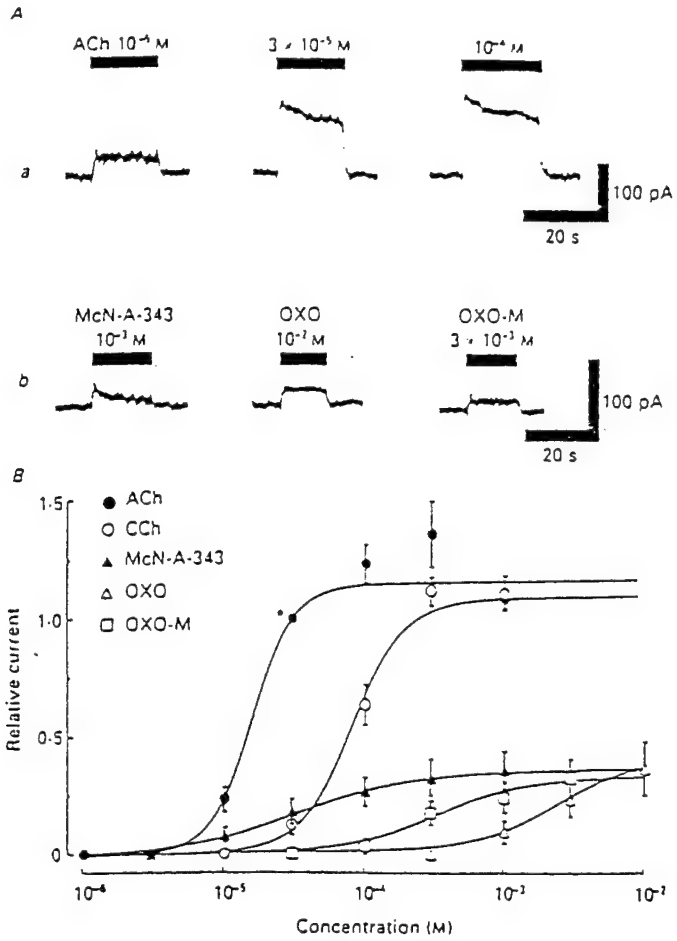


Figure 2–12. I_{ACh} in dissociated outer hair cells (OHCs). A a,I_{ACh} at various concentrations under voltage-clamp conditions using the perforated patch-clamp technique. The holding potential (V_H) was –40 mV. Horizontal bars above each response indicate a period of continuous ACh application. The current was recorded from a 40 μm OHC of the second turn. The resting membrane potential (VR) and the input impedance were –60 mV and 33 MΩ, respectively. The amplitude of the current injected to hold the membrane potential of –40 mV was 600 pA. A b, representative current induced by McN-A-343, oxotremorine (OXO), and oxotremorine-M (OXO-M). The 50 μm cell was obtained from the second turn. The V_R and the input impedance were –62 mV and 67 MΩ, respectively. The amplitude of the current injected to hold the membrane potential of –40 mV was 320 pA. B, concentration-response relationships for ACh, carbamylcholine (Cch), and various muscarinic agonists. Amplitudes of currents induced by each drug at various concentrations were normalized to the current induced by 3×10^{-5} M ACh (*). Each point is the mean ± S.E.M. of four to seven cells. (From Kakehata et al., 1993, with permission.)

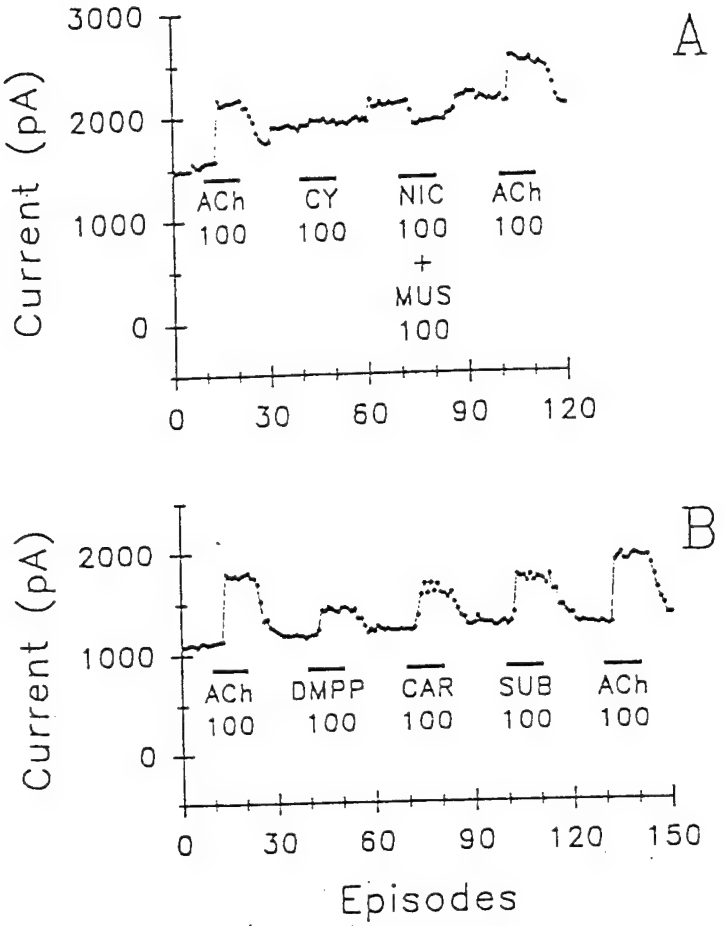


Figure 2–13. Effect of the ACh receptor agonists compared to ACh in two cells; frame A, cytisine (CY) and nicotine with muscarine (NIC + MUS); frame B: DMPP, carbachol (CAR) and suberyldicholine (SUB). In frame A between records (every 30 episodes) an apparent recovery of some current strength occurred as in Figure 2–8. Note the mixture of nicotine with muscarine suppressed the current. Numbers near the drug abbreviation are drug concentrations in μM. Shown are data obtained as voltage clamp current responses at zero mV utilizing the same step protocol as illustrated and described in Figure 2–2. See Figure 2–2 for further description. (From Erostegui et al., 1994, with permission.)

TABLE 2-3. Summary of the various antagonists of ACh at the OHC and a list of the other receptors where these antagonists are thought to be most selective.

Receptor Type[a]	Agonist	Location	ACh Antagonist[b]
suberyldicholine or subdic (S)	Ach, suberyl-dicholine	OHCs	strychnine
nicotonic (Nm)	ACh, nicotine	skeletal muscle	curare, α-BTX
nicotinic (Nn)	ACh, nicotine	autonomic ganglia	trimethaphan
muscarinic (M)	ACh, muscarine	smooth muscle glands	atropine
muscarinic (M_1)	ACh, muscarine	"	pirenzepine
muscarinic (M_2)	ACh, muscarine	"	AF-DX 116
muscarinic (M_3)	ACh, muscarine	"	4-DAMP
glycinergic (gly)	glycine	central nervous system	strychnine
GABAergic (GABA)	GABA	"	bicuculline

[a] Suberyldicholine is the name proposed for the ACh receptor type on the OHCs.
[b] All of the antagonists listed block the effects of ACh at the OHC. Only strychnine is listed at the OHC because it is the most potent antagonist against ACh.

belongs. It is unlikely that ACh belongs to a new family, even though new families of receptors are being discovered (e.g., ATP family; Valera et al., 1994). However, until the molecular composition of the subdic receptor is determined, the "family" will remain unknown.

Adenosine triphosphate (ATP)

ATP was first suggested to be a candidate for a neurotransmitter or neuromodulator in the cochlea based on its relative potency in reducing the compound action potential of the auditory nerve (Bobbin & Thompson, 1978). Subsequent studies have demonstrated that ATP increases intracellular Ca^{2+} levels in inner hair cells (Dulon, Mollard, & Aran 1991) and Deiters cells (Dulon et al., 1993) and has powerful effects on the membrane potential of the OHCs (Ashmore & Ohmori, 1990; Housley, Greenwood, & Ashmore, 1992; Kakehata et al., 1993; Kujawa, Erostegui, Fallon,

Crist, & Bobbin, 1994b; Nakagawa, Akaike, Kimitsuki, Komune, & Arima, 1990). ATP activates a receptor on OHCs that induces a large inward current and so depolarizes the OHC.

ATP is metabolized very rapidly to adenosine, a compound with little activity in the cochlea. However, chemists have made a few ATP analogues that are not as rapidly metabolized, such as ATP-γ-S. Recently, Kujawa et al. (1994b) demonstrated that ATP-γ-S was one of the most potent compounds studied when instilled into the perilymph: ATP-γ-S abolished the CAP and the DPOAEs, and decreased the low intensity sound-evoked SP while it increased the high intensity sound-evoked SP (Figure 2–14). In addition, Kujawa, Fallon, and Bobbin (1994c) demonstrated powerful effects of ATP antagonists on cochlear potentials suggesting a role for endogenously released ATP in normal physiology.

The role of ATP and its receptor proteins in the cochlea is unknown. It may have both a paracrine and an autocrine role. Some suggest ATP is released from the efferent nerve fibers and as such is utilized as a depolarizing agent to counter the hyperpolarizing effects of ACh. Others suggest it may be acting on the scala media side of the OHC (near the stereocilia) to regulate the polarization of the OHC (Housley et al., 1992). In addition, ATP may have a role in programmed cell death (Valera, Hussy, Evans, Adami, North, Surprenant, & Buell, 1994). In summary, ATP has powerful effects in the cochlea, but the role of these effects in the physiology of the cochlea remains to be determined.

Other chemicals

GABA appears to be a transmitter at a small number of efferent nerve fibers that synapse on OHCs (see review by Eybalin, 1993). GABA may allow ions such as chloride to enter the cell and so hyperpolarizes the OHC (Gitter & Zenner, 1992).

Additional chemicals have been suggested to have a possible role at the OHCs. Glutamate, which is the transmitter between IHCs and the afferent nerves, and probably the transmitter between the OHCs and their afferent nerves (Bobbin, 1991) when applied acutely has no effect on the currents recorded from the OHCs in the whole cell voltage clamp configuration (Chen & Bobbin, 1994). However, glutamate may have a role on second messenger chemicals inside the OHCs that we cannot detect by measuring acute changes in cell current. Calcitonin-gene-related-peptide (CGRP) is another chemical found in the efferents innervating the OHCs for which a role has yet to be determined (Eybalin, 1993).

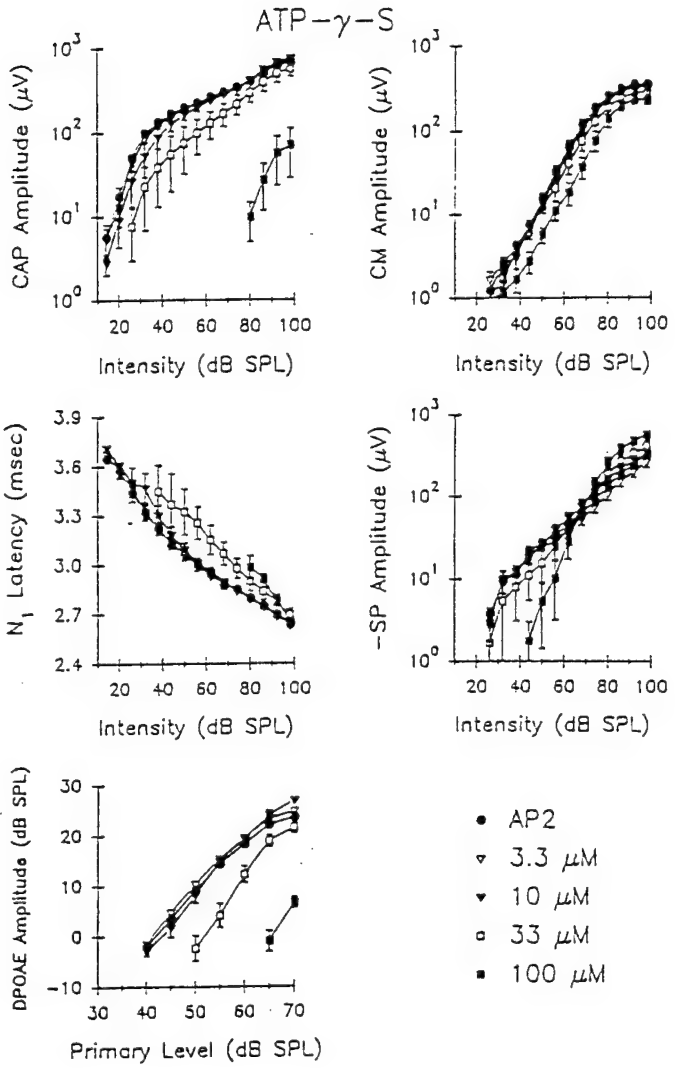
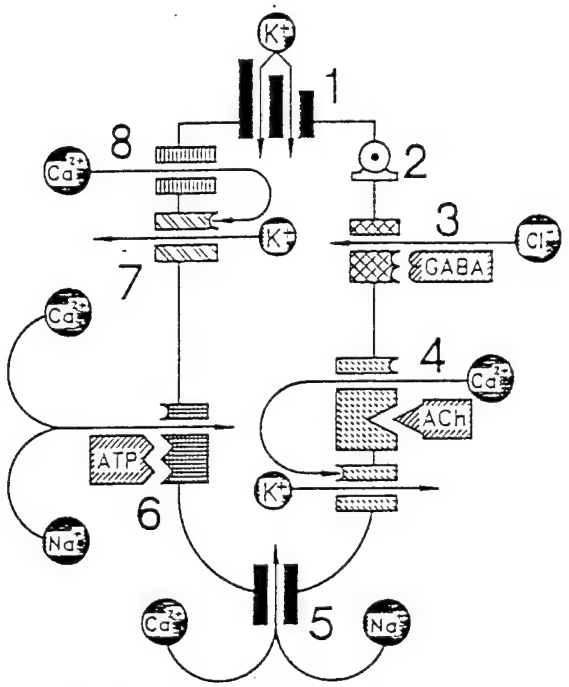


Figure 2–14. Effect of ATP-γ-S on CAP, N1 latency, SP, CM, and DPOAE responses as a function of stimulus intensity. Shown are functions recorded after pre-drug artificial perilymph perfusion No. 2 (AP2) and after perfusion with increasing concentrations (3.3–100 μM) of ATP-γ-S. Data are represented as means ± S.E. across 5 animals. (From Kujawa et al., 1994b, with permission.)

RECEPTOR MECHANISMS

The action of these chemicals at the level of the OHC is summarized in Figure 2–15. The movement of the stereocilia opens the transduction channel allowing potassium ions into the cell which results in depolarization of the OHC (see review by Roberts, Howard, & Hudspeth, 1988). This activates the motor protein in the OHC membrane that causes the



1. transduction channel
2. motor
3. GABA–receptor & ion channel complex
4. ACh–receptor & ion channel complex
5. non–specific cation channel
6. ATP–receptor & ion channel complex
7. K_{Ca} and K_n channel
8. L–type Ca^{2+} channel

Figure 2–15. Model of an OHC illustrating the various ion channel proteins in the membrane and available to alter the membrane potential.

length change in the cell, the motor being sensitive to the voltage of the cell (depolarization or voltage change in a positive direction = decrease in length; hyperpolarization or voltage change in a negative direction = increase in length).

In Figure 2–15 I have represented three types of ion channels that will respond to voltage changes in the cell: a nonspecific cation channel, an L-type Ca^{2+} channel, and at least two types of Ca^{2+}-activated potassium channels which are represented in Figure 2–14 as one channel (Housley & Ashmore, 1992; Nakagawa et al., 1991). These ion channels are voltage-activated channels, that is, they will open in response to voltage changes and allow certain ions to move across the membrane of the OHC. The ion flows they allow will in turn change the voltage of the cell. GABA, ACh, and ATP, which are called "ligands," act on receptor proteins that regulate or open other ion channels and, therefore, these channels are called "ligand-gated channels." Ligand-gated channels are usually not opened by voltage changes. Ligands can act on the receptor protein which is an actual part of the ion channel or they can act on receptor proteins that interact with G proteins, which in turn can act on ion channels and other enzymes in the cell. In the latter case the mechanism of receptor action is called metabotropic and in the former case it is called ionotropic.

Whether the subdic receptor protein is metabotropic or ionotropic is currently being debated. As mentioned previously, Housley and Ashmore were the first to demonstrate that, in mammalian cochlear OHCs, ACh induces an opening of a channel (K_{ACh}) that allows K^+ ions to move across the membrane of the OHC, down its electrical and chemical gradient. At membrane potentials positive to –70 mV, K^+ will move out of the OHC and result in a hyperpolarization of the cell. Experimentally, the opening of the K_{ACh} channel induced by ACh requires the presence of both extracellular and intracellular free Ca^{2+}. Therefore, most researchers feel that the opening of the K_{ACh} channel by ACh involves an intermediate step which increases the level of free Ca^{2+} inside the cell adjacent to the K_{ACh} channel. This intermediate step may consist of ACh directly opening an ion channel linked to the receptor that allows Ca^{2+} to enter the cell, as illustrated in the Figure 2–15 (Fuchs & Murrow, 1992a). In contrast, Kakehata et al. (1993) suggest that ACh stimulates phosphatidylinositol metabolism via a PTX-sensitive G-protein. The phosphatidylinositol then releases Ca^{2+} from intracellular stores to act on the KACh channel. Such a mechanism is illustrated in Figure 2–16. So the major immediate question that needs to be answered is what is the mechanism of the subdic receptor: Does the receptor act by opening a Ca^{2+}-selective cation channel (ionotropic) or does it activate a G protein (metabotropic)?

In the OHCs, ATP appears to open a cation selective channel to allow sodium and Ca^{2+} into the cell (depolarization) as shown in Figure 2–15.

Others suggest that ATP may also activate the G-protein coupled to the phosphoinositol cascade in a manner similar to ACh, as shown in Figure 2–16 (Niedzielski & Schacht, 1992).

GABA appears to open an ion channel permeable to the chloride ion. This would hyperpolarize the cell similar to the effects of acetylcholine.

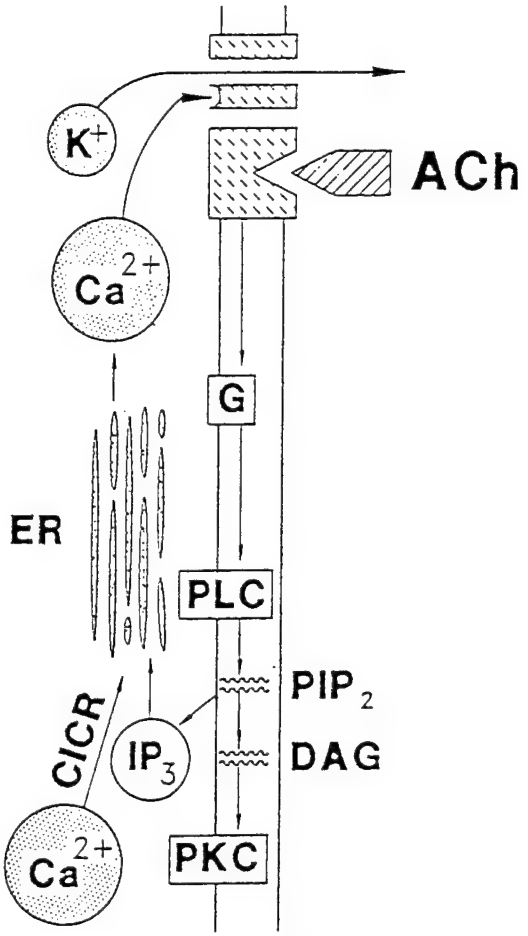


Figure 2–16. An alternative model (metabotropic) of the ACh receptor\ion channel complex illustrating a possible role for G protein and ionositol triphosphate (IP_3) in providing the intracellular Ca^{2+} for opening the K^+ channel. G: g protein; PLC: phospholipase C; PIP_2: phosphoinositol bisphosphate; DAG: diacylglycerol; PKC: phosphokinase C; CICR: calcium-induced calcium release; ER: endoplasmic reticulum.

In summary, these voltage-sensitive and ligand-activated channels in concert or alone can modify the voltage of the cell and the cells' length and length-change response to sound. In this manner, the active process is regulated and modulated. When and how all these channels are orchestrated to yield a functional active process awaits additional research.

Acknowledgments: The author wishes to thank Maureen Fallon and Jim Bolongaro for drawing the illustrations and Anastas Nenov, Chu Chen, Latasha Bright, Elaine McDonald, Cristopher LeBlanc, and Ruth Skellett for help. This work was funded in part by the following: NIH research grants DC00722, DC00379, and DAMD 17-93-V-3013, Kam's Fund for Hearing Research, and the Louisiana Lions Eye Foundation.

GLOSSARY

Autocrine. A chemical substance, also known as messenger, that is released from cells and reaches targets (receptors) located on the same cell by diffusion, for example, autoneurotransmitters.

Depolarization. Change in the resting membrane potential of a cell in the positive direction, less negative.

Hyperpolarization. Change in the resting membrane potential of a cell in the negative direction, less positive.

Ionotropic. A receptor mechanism for a neurotransmitter: the receptor protein is part of an ion channel\receptor protein complex and opens or closes the ion channel directly in response to the presence of a ligand (i.e., any compound that binds to a receptor) for that receptor.

Metabotropic. A receptor mechanism for a neurotransmitter: the receptor protein acts via another protein such as a g protein to activate or inhibit enzymes and possibly open, close, or modify ion channels.

Muscarinic receptor protein. The receptor activated by the neurotransmitter, acetylcholine (ACh), and the drug, muscarine, located at the autonomic innervation of glands, cardiac muscle, and smooth muscle—abbreviated M.

Neurotransmitter. Chemical released from a sensory receptor cell or nerve cell on depolarization of that cell. On release the chemical diffuses across the gap between the releasing cell and an adjoining cell to act on the adjoining cell to induce a change in its electrical or chemical properties.

Nicotinic receptor protein. The receptor activated by the neurotransmitter, acetylcholine (ACh), and by the drug, nicotine, located at the neuromuscular junction of skeletal muscle—abbreviated Nm.

Paracrine. A chemical substance, also known as a messenger, that is released from cells and reaches different target cells by diffusion, for example, neurotransmitters.

Receptor protein. Protein in the membrane of a cell that accepts a chemical messenger (ligand) such as the neurotransmitter, changes its configuration on accepting the neurotransmitter, and so induces subsequent reactions in the cell, such as opening of an ion channel to allow for diffusion of that ion down its concentration and electrical gradient.

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Appendix #5
DAMD17-93-V-3013
Bobbin and Berlin

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Hearing Research 98 (1996) 68–76

HEARING RESEARCH

Chronic low-level noise exposure alters distortion product otoacoustic emissions

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Received 11 October 1995; revised 13 March 1996; accepted 17 March 1996

Abstract

Chen et al. (1995) recently reported an altered response to the application of ATP in outer hair cells (OHC) isolated from guinea pigs continuously exposed for 10 or 11 days to a 65 dB SPL (A-scale) narrow-band noise (1.1–2.0 kHz). The primary goal of the present study was to test the hypothesis that the continuous low-level noise used by Chen et al. (1995) alters cochlear function. Cubic ($2f_1$-f_2) and quadratic (f_2-f_1) DPOAEs, as well as, the amount of contralateral suppression of DPOAE amplitudes were chosen for study. Responses were recorded in urethane-anesthetized guinea pigs with sectioned middle ear muscles. The animals had either been exposed to the low-level noise for 3 or 11 days or not exposed at all ($n = 13$ animals per group). Results demonstrate that this noise induces frequency-dependent and very localized reductions in $2f_1$-f_2 DPOAE input/output (I/O) functions. However, the f_2-f_1 DPOAE I/O functions appear to be insensitive to the noise exposure. No noise-related changes were found in the amount of contralateral suppression between the different exposure groups, with the exception of one unexplainable data point (f_2-f_1 DPOAE = 0.5 kHz; day 3) where it was reduced. The $2f_1$-f_2 DPOAE amplitude alterations lend support to the conclusions of Chen et al. (1995) that chronic low-level noise exposure induces molecular changes in the OHCs which may, in turn, alter cochlear function.

Keywords: Noise exposure; Distortion product otoacoustic emission; Outer hair cell

1. Introduction

Sound exposure induces changes in the structure and function of the auditory pathway (Saunders et al., 1985, 1991). These changes depend on the duration, intensity, and temporal pattern of the exposure. Continuous sound exposure produces more profound effects on the auditory system than interrupted sound exposure of equal acoustic energy (Ward, 1976, 1991; Bohne et al., 1985, 1987; Clark et al., 1987; Clark, 1991; Fredelius and Wersäll, 1992). The alterations in auditory function after very intense levels of sound can be attributed to mechanical changes in cochlear structure. On the other hand, low levels of sound produce complex changes within the cochlea that are not well described.

Above a certain level, continuous noise exposure will result in a decrease in auditory sensitivity. This decrease in sensitivity becomes larger during the first 24 h of exposure and then plateaus as the exposure continues, remaining stable during exposures lasting as long as 3 years (Clark and Bohne, 1987). This pattern of sensitivity change is referred to as an asymptotic threshold shift (ATS). ATS has been demonstrated in chinchillas (Carder and Miller, 1972; Mills, 1973, 1976; Bohne and Clark, 1982; Clark and Bohne, 1987), monkeys (Moody et al., 1976), guinea pigs (Syka and Popelar, 1980), and humans (Melnick, 1976). The level and duration of noise exposure determines whether ATS will fully recover (temporary threshold shift, TTS) or have a permanent component (permanent threshold shift, PTS; see Fig. 1 in Drescher, 1976).

The minimum level of continuous noise exposure required to produce a threshold shift depends upon the band of noise used and the species of animal under investigation. These minimum levels have been fairly well worked out in chinchillas for octave bands of noise centered at 0.5 and 4.0 kHz. Carder and Miller (1972) suggested that a 0.5 kHz octave band noise should not induce an elevation of

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PII S0378-5955(96)00062-7

auditory threshold when the exposure level is at or below 55 dB SPL. This was supported by Bohne (1976) who observed no structural damage to chinchilla hair cells after 9 days of exposure to a noise of 65 dB SPL and the same spectral characteristics. For an octave band of noise centered at 4.0 kHz, Mills (1973) suggested that a level at or below 47 dB SPL should not produce ATS. According to these results, as the center frequency of the noise band increases, the exposure level required to produce ATS decreases.

To date, there is little, if any, information about the minimum level of continuous noise required to produce a threshold shift in guinea pigs. Davis et al. (1935) exposed nine guinea pigs to a 600 Hz pure tone at 65 dB for 70 days and found slight losses in sensitivity in only one of the animals. Canlon and Fransson (1995) exposed guinea pigs to a 1.0 kHz pure tone at 81 dB SPL for 24 days. They reported that this exposure did not cause any significant changes in auditory brainstem response thresholds and DPOAE amplitudes of the noise-exposed animals as compared to the unexposed animals. Syka and Popelar (1980) demonstrated the existence of an ATS over the course of a 5 day exposure to a 100 dB SPL third octave band of noise centered at 2.0 kHz. However, the authors did not explore the minimum level of noise necessary because a significant PTS was still present 120 days post-exposure.

Chen et al. (1995) reported that a 10 or 11 day exposure to a 65 dB SPL (A-scale) narrow-band noise (1.1–2.0 kHz) altered the response of guinea pig isolated OHCs to ATP application. This level of noise is very close to the minimum levels suggested to induce changes that would be observed as a threshold shift in chinchillas exposed to an octave band noise centered at 0.5 kHz (Carder and Miller, 1972). Thus, it is possible that the particular noise used by Chen et al. (1995) changes auditory threshold. To partially address this question, we asked whether the noise used by Chen et al. changes cochlear function? Puel et al. (1988, 1995) demonstrated that a low level of intense sound affects the active process before it affects the passive process. Thus there is a high probability that since the noise used by Chen et al. was very low, it only affected the active process. Others, utilizing salicylate, have demonstrated that the active process is integrally linked to OHC function and low level cochlear mechanics (Puel et al., 1989; Shehata et al., 1991; Kujawa et al., 1992). Therefore, to answer the question raised by the study of Chen et al., we examined the effects of the chronic low-level noise exposure on the cubic DPOAE ($2f_1$-f_2) and the quadratic DPOAE (f_2-f_1). DPOAE measurements were chosen for study since they are believed to reflect the status of OHC function (Siegel and Kim, 1982; Siegel et al., 1982). Others have speculated that ATP may be released by the efferents synapsing on the OHCs (e.g., Eybalin, 1993), therefore, the response reported by Chen et al. may be detected as an alteration in the effectiveness of efferent neurons. This was monitored by the amount of contralateral suppression of the DPOAEs (Puel and Rebillard, 1990; Kirk and Johnstone, 1993; Kujawa et al., 1993).

2. Methods

2.1. Subjects

Pigmented guinea pigs ($n = 39$) of either sex (300–500 g) were used as subjects. Upon delivery from the supplier, animals were randomly assigned to three groups ($n = 13$ per group) according to the number of days exposed to a low-level continuous noise (0, 3, and 11 days). The control group was not exposed to the noise. These animals were housed at the university's animal care facility. The experimental groups were exposed to a 65 dB SPL (A-scale) narrow-band noise (cutoff frequencies 1.1 and 2.0 kHz) 24 h/day for 3 or 11 days. These animals were tested approximately 1.5 h after removal from the noise because of the time necessary for the surgical procedures described below. All animals were given free access to food and water. The care and use of the animals that were used in this study were approved by LSUMC's Institutional Animal Care and Use Committee.

2.2. Noise exposure facility and noise generation

Unanesthetized guinea pigs were exposed to the continuous noise in groups of 10 or less in a small sound-attenuated booth. A small light was installed within the booth and controlled with a timer to provide the animals 12 h of light and 12 h of darkness.

The noise was generated by a WG2 Waveform Generator (Tucker-Davis Technologies) which was set to produce a uniform noise signal. This signal was filtered using a Brickwall Filter (Wavetek/Rockland Model 753A) configured in the bandpass mode with a low-frequency cutoff at 1.1 kHz and a high-frequency cutoff at 2.0 kHz. The level of the noise was controlled by a PA4 Programmable Attenuator (Tucker-Davis Technologies). Additional power was gained using a power amplifier (McIntosh MC2100) producing the final signal delivered to the speaker (Realistic 40-1286C; 8Ω, 30 W).

Noise levels were monitored by placing a sound level meter (Brüel & Kjær Type 2230 Precision Integrating Sound Level Meter) within the booth. The microphone was positioned so that it was approximately at the level of the guinea pigs' ears and was placed at various positions around the booth to ensure that the noise was equally distributed throughout the booth. The spectrum of the noise as measured with a signal analyzer (Hewlett-Packard 3561A) is shown in Fig. 1.

2.3. General surgical methods

Immediately prior to testing, the animals were anesthetized by administering a dose of urethane (Sigma; 1.5

g/kg, i.p.), tracheotomized, and allowed to breathe unassisted. Supplementary doses of anesthetic (urethane, 1.5 g/kg) were given to maintain an adequate depth of anesthesia. Electrocardiogram and rectal temperature were monitored throughout each experiment and temperature maintained at 38 ± 1°C using a heating pad.

The surgical procedures used were similar to those described by Kujawa et al. (1993). Briefly, cartilaginous ear canals were exposed and partially removed to allow for proper placement of hollow ear bars which help secure the animal in the headholder. This procedure also ensured optimal coupling of the sound delivery system to both ears. Using a ventrolateral approach, the ipsilateral (right) auditory bulla was exposed and opened to gain access to the tendons of the middle ear muscles. These tendons were sectioned in all animals to prevent the involvement of middle ear muscle contraction on DPOAE measurements.

2.4. DPOAE generation and measurement

Equilevel primary tones, f_1 and f_2, were generated under computer control using Tucker-Davis System 2 audio processing equipment. More specifically, the computer generated primaries were sent to two separate channels of a DA1 digital-to-analog converter and attenuated to desired levels using PA4 programmable attenuators. The analog signals were then sent to two separate channels of an FT5 anti-aliasing low-pass filter with a 20 kHz cutoff frequency and then to an HB5 headphone buffer before being sent to two separate speakers (Etymotic Research, ER-2) housed within an acoustic probe assembly. The acoustic probe assembly was tightly coupled to the right ear of each animal. DPOAEs were detected by a sensitive microphone (Etymotic Research, ER-10) also housed within the probe assembly and amplified using a microphone preamplifier (Etymotic Research, ER-1072). A dynamic signal analyzer (Hewlett-Packard, 3561A) was used

Table 1
Primary tones (f_1,f_2) and corresponding cubic ($2f_1$-f_2) and quadratic (f_2-f_1) DPOAEs (in kHz)

f_1	f_2	f_2-f_1	$2f_1$-f_2
1.250	1.500	–	1.000
1.563	1.875	0.312	1.250
2.500	3.000	0.500	2.000
3.750	4.500	0.750	3.000
5.000	6.000	1.000	4.000
6.250	7.500	1.250	5.000
7.500	9.000	1.500	6.000
10.000	12.000	2.000	8.000

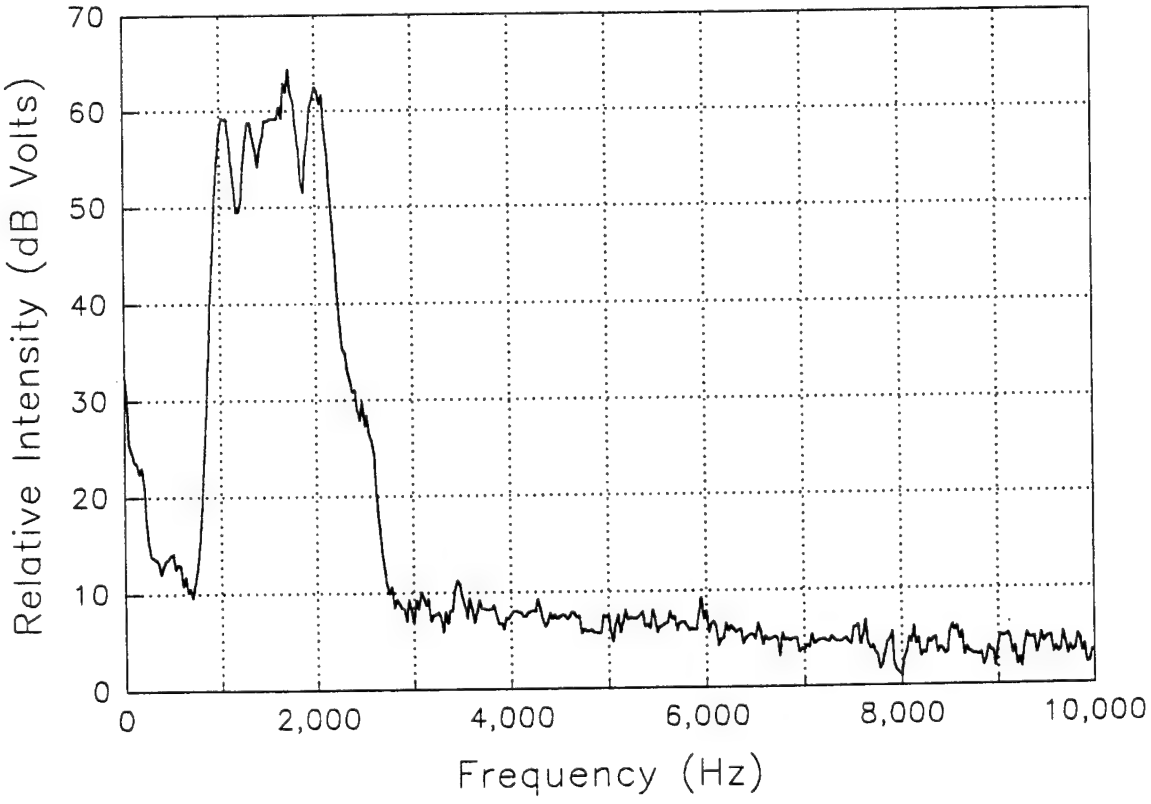


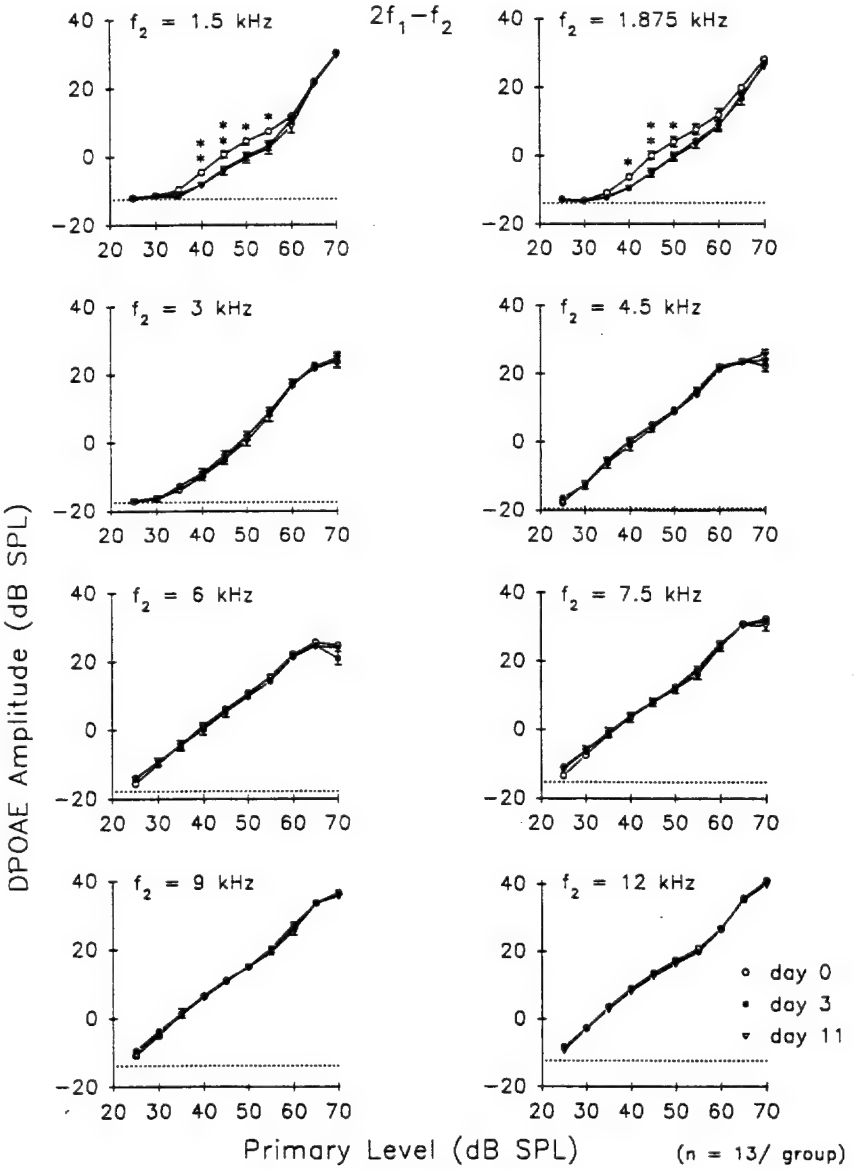
Fig. 1. The frequency spectrum of the 65 dB SPL (A-scale) noise used for continuous exposure. The sound level was monitored with a sound level meter and a 1\2 in. microphone (Brüel & Kjær type 2230 meter and type 4155 microphone) and viewed with a dynamic signal analyzer (Hewlett-Packard 3561A) set at a bandwidth of 95.485 Hz.

average the DPOAE responses for FFT analysis and ıectral display (CF = DPOAE frequency; span = 1 kHz; W = 3.75 Hz).

DPOAE amplitudes were measured for several combiıtions of f_1 and f_2 having a ratio $f_2/f_1 = 1.2$ (Table 1). his ratio is within the range describing the optimal freıency separation of f_1 and f_2 for the guinea pig (1.2–1.3; rown, 1987; Brown and Gaskill, 1990). Since the place origin of the DPOAEs is believed to be near, or at, the place on the cochlear partition (Matthews and Molnar, 986; Brown et al., 1992; Allen and Fahey, 1993), all data e expressed as a function of f_2 rather than the frequency the DPOAE (Table 1). DPOAEs were elicited with ıuilevel primaries tones ($L_1 = L_2$). Both quadratic (f_2-f_1) ıd cubic ($2f_1$-f_2) DPOAEs were studied. The primary tones were presented in descending order, starting at a level of 70 dB SPL and decreasing in 5 dB steps to 20 dB SPL. DPOAE I/O functions (i.e., plots of primary level vs. DPOAE amplitude for each f_2) were generated.

2.5. Contralateral noise generation

The 70 dB SPL wide-band noise delivered to the contralateral (left) ear to activate the efferents was also generated under computer control using Tucker-Davis System 2 audio processing equipment. In particular, a W1 Waveform Generator was set up to produce a uniformly distributed noise signal. The level of the was controlled with a PA4 Programmable Attenuator and then sent to a speaker (Etymotic Research, ER-2). A polyethylene tube (1.35 mm



g. 2. Effect of continuous low-level noise exposure on $2f_1$-f_2 DPOAE I/O functions for different frequencies of f_2. Shown are data (means ± SE; = 13 animals/group) for the unexposed group (day 0; ○), the group of animals exposed for 3 days (day 3; ●), and the group of animals exposed for 11 ıys (day 11; △). * $P < 0.05$ and ** $P < 0.01$. The dashed line represents the noise floor.

ID, 292 mm length) attached to the output of the speaker was used to deliver the noise down the hollow ear bar coupled to the entrance of the bony canal of the left ear. The spectrum of the noise presented to the contralateral ear was flat (±10 dB) from 0.9 to 15.8 kHz and rolled off approximately 50 dB/octave above 15.8 kHz.

2.6. Contralateral suppression measurements

DPOAEs were recorded, both with and without the wide-band noise (70 dB SPL) presented to the contralateral ear for several combinations of f_1 and f_2 ($f_2/f_1 = 1.2$; $L_1 = L_2 = 60$ dB SPL). Table 1 lists the primary frequencies and the corresponding DPOAEs (f_2-f_1 and $2f_1$-f_2) that were studied. The paradigm used to measure the amount of contralateral suppression is the same as that used by Kujawa et al. (1995). Briefly, five consecutive averages of DPOAE amplitude (10 spectra per averages) were first obtained in the absence of contralateral stimulation. The wide-band noise was then delivered to the contralateral ear and again five consecutive 10-spectra averages of the DPOAE amplitude were obtained. The final five consecutive averages were taken after the contralateral stimulation was removed. Each of these averages required approximately 5 s to complete. A total of 25 s of amplitude monitoring was obtained for each condition.

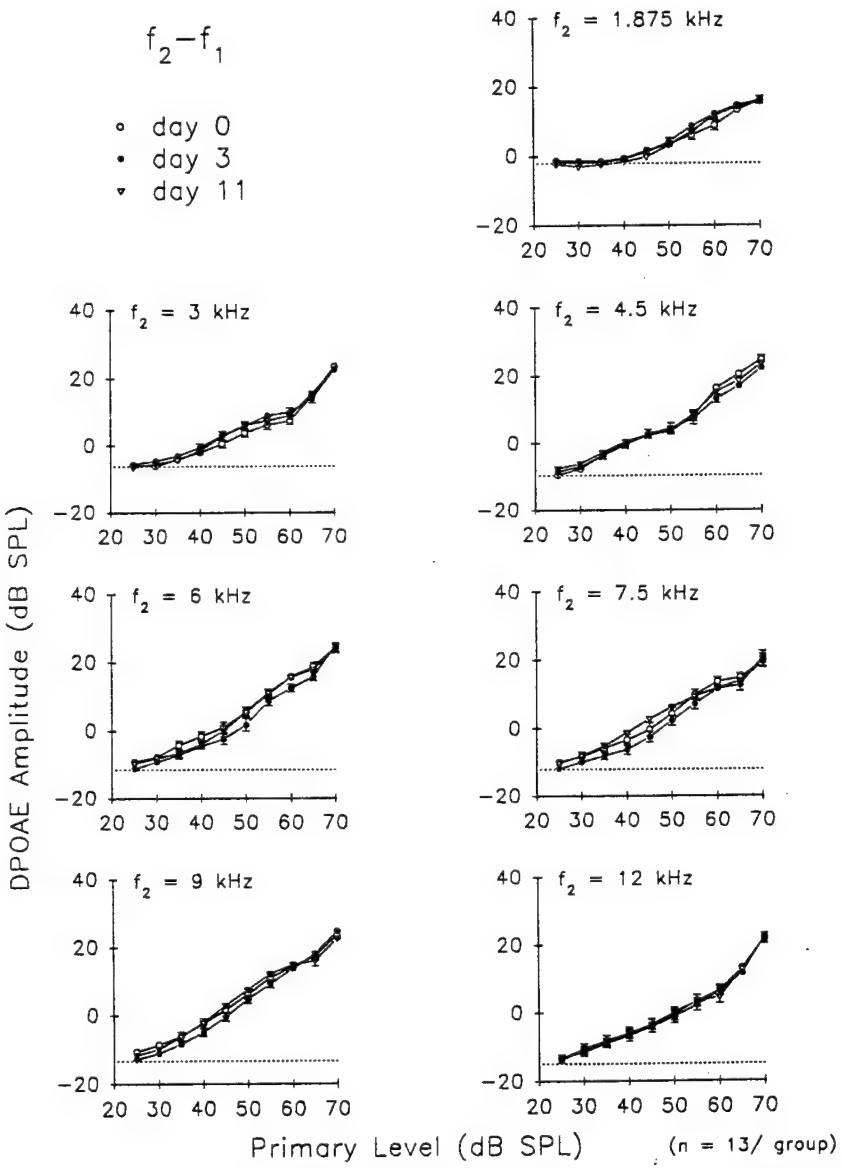
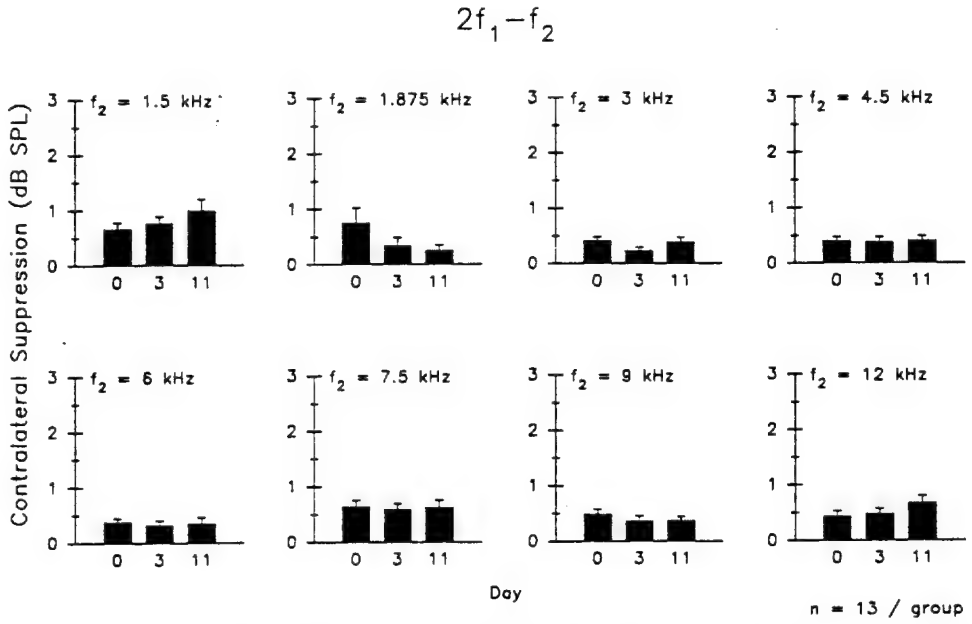


Fig. 3. Effect of continuous low-level noise exposure on f_2-f_1 DPOAE I/O functions for different frequencies of f_2. Shown are data (means ± SE; $n = 13$ animals/group) for the unexposed group (day 0; ○), the group of animals exposed for 3 days (day 3; ●), and the group of animals exposed for 11 days (day 11; △). The dashed line represents the noise floor.



ig. 4. Effect of continuous low-level noise exposure on the amount of contralateral suppression for the $2f_1$-f_2 DPOAE for different frequencies of f_2. POAEs were monitored before (5 trials) and during (5 trials) presentation of wideband noise (70 dB SPL) to the contralateral ear. Each trial represents a) spectra average and required 5 s to complete. The bars represent the amount of contralateral suppression which was calculated by subtraction the mean f the 5 during trials from the mean of the 5 before trials. Data are represented as means ± SE (n = 13 animals/group).

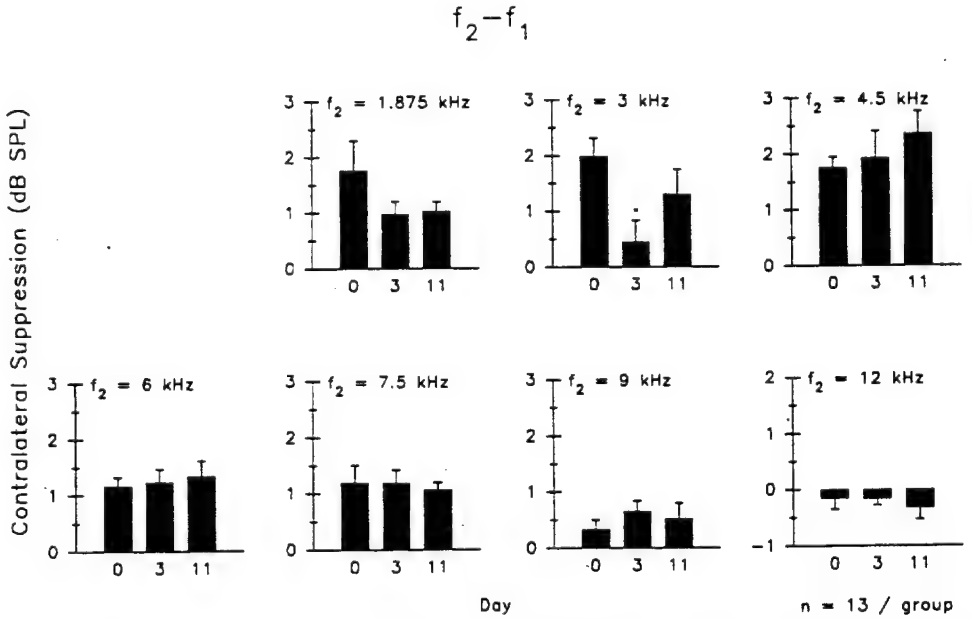
.7. Data analysis

Data are presented as means ± SE. Effects of the low-evel noise exposure on DPOAE I/O functions and con-alateral suppression were quantified using 1-way (by xposure group) analysis of variance (ANOVA) and New-nan-Keuls post-hoc tests. P values less than 0.05 were onsidered statistically significant.

3. Results

3.1. Effect of continuous low-level noise exposure on DPOAE I/O functions

Fig. 2 illustrates the $2f_1$-f_2 DPOAE I/O functions of the unexposed (control) guinea pigs and the guinea pigs exposed continuously to the 65 dB SPL narrow-band noise



ig. 5. Effect of continuous low-level noise exposure on the amount of contralateral suppression for the f_2-f_1 DPOAE for different frequencies of f_2. See ig. 4 for additional information.

for either 3 or 11 days. The results show significant differences between the control group and both noise-exposed groups in the DPOAE I/O functions elicited when f_2 was in the region of the noise band (Fig. 2; $f_2 = 1.5$ kHz and 1.875 kHz). Only intensity levels above the noise floor and ranging from 40 to 55 dB SPL were significantly different (Fig. 2). All other DPOAE I/O functions were not significantly different (Fig. 2; $f_2 = 3.0$, 4.5, 6.0, 7.5, 9.0, and 12.0 kHz; $P > 0.05$).

Fig. 3 illustrates the f_2-f_1 DPOAE I/O functions of the unexposed (control) guinea pigs and the guinea pigs exposed continuously to the 65 dB SPL narrow-band noise for either 3 or 11 days. The results did not show any significant differences in the DPOAE I/O functions between any of the groups tested for any combination of primary tones studied ($P > 0.05$).

3.2. Effect of continuous low-level noise exposure on contralateral suppression

Fig. 4 and Fig. 5 illustrate the average ($n = 13$ animals/group) amount of contralateral suppression measured in the unexposed group of animals and the groups of animals exposed to the 65 dB SPL narrow-band noise for both the $2f_1$-f_2 and f_2-f_1 DPOAE. The only suppression that was statistically different from day 0 was the 0.5 kHz f_2-f_1 DPOAE ($f_2 = 3$ kHz) at day 3 (Fig. 5). Other than for this one measure, there were no other statistically significant changes in the amount of contralateral suppression over the course of the continuous noise exposure in either the f_2-f_1 or $2f_1$-f_2 DPOAEs.

4. Discussion

4.1. Level of noise exposure and threshold

Chen et al. (1995) reported that a 65 dB SPL narrow-band noise (1.1–2.0 kHz) altered the response of OHCs to ATP. The primary goal of the present study was to test the hypothesis that the continuous low-level noise used by Chen et al. alters cochlear function. Results demonstrate that this noise induces a frequency-dependent and very localized reduction in $2f_1$-f_2 DPOAE I/O functions in guinea pigs exposed continuously for 3 and 11 days.

To the best of our knowledge, the level used in the present study is the lowest level of noise found to produce changes in the auditory function of the guinea pig. It is interesting to note that there were similar reductions in DPOAE amplitude for the groups of animals exposed for 3 and 11 days. This pattern of amplitude change resembles that of an ATS (Carder and Miller, 1972). There are no previous reports of 'ATS-like' changes in DPOAE amplitude in response to long-term noise exposure. However, we only monitored the changes in DPOAE amplitude on 2 days during our noise exposure protocol. Therefore, more experiments will have to be performed to characterize the time course of DPOAE amplitude change and to determine the degree of PTS and TTS.

4.2. Noise-induced effects on OHCs and/or cochlear mechanics

Significant amplitude reductions of the $2f_1$-f_2 DPOAE occurred only when the frequency of f_2 was within the noise exposure band. This finding is consistent with the belief that the f_2 place along the cochlear partition is the generation site of $2f_1$-f_2 DPOAEs (Matthews and Molnar, 1986; Brown et al., 1992; Allen and Fahey, 1993; Puel et al., 1995). Damage to this place results in the reduction and possibly complete loss of the corresponding $2f_1$-f_2 DPOAE (Siegel and Kim, 1982; Siegel et al., 1982; Zurek et al., 1982). Further, the changes in DPOAE amplitude induced by the noise exposure occurred only in the region of the I/O functions elicited by low-to-moderate level primaries. These low-level DPOAEs are thought to be mediated by active cochlear mechanics and are physiologically vulnerable to the same factors that normally damage or destroy OHC function.

We examined the effect of the noise on the f_2-f_1 DPOAE, since it is thought that these events reflect a different mechanical process in the cochlea then does the $2f_1$-f_2 DPOAE (Brown, 1988). Results show that there was no significant change in the amplitude of the f_2-f_1 DPOAE I/O functions even when the f_2 frequency was within the noise band. These results appear to indicate that the f_2-f_1 DPOAE are insensitive to the noise exposure used in our study.

4.3. ATP and efferents

Although speculative, it has been suggested that the cochlear efferents may release ATP onto the OHCs (Nakagawa et al., 1990; Eybalin, 1993). However, to date no response observed upon efferent activation has been attributed to released ATP. Nevertheless, it is possible that the reduction of the ATP response in the isolated OHCs reported by Chen et al. (1995) may decrease the effectiveness of the efferent innervation on the OHCs. Therefore, we examined effects of the noise exposure on the amount of DPOAE amplitude suppression obtained during presentation of sound to the contralateral ear, an effect known to be efferent-mediated (Warren and Liberman, 1989; Puel and Rebillard, 1990; Kirk and Johnstone, 1993; Kujawa et al., 1993). With the exception of one unexplainable data point (f_2-f_1 DPOAE = 0.5 kHz; day 3) where it was reduced, no additional changes were found in the amount of contralateral suppression. This failure to detect a change may be due to the fact that the ATP may not play a role in this efferent phenomena.

‹. Summary

In summary, results show that the chronic noise expo-
e used in this study and by Chen et al. (1995; 65 dB
L, 1.1–2.0 kHz) does, in fact, alter cochlear function as
asured by changes in the $2f_1$-f_2 DPOAE. The amplitude
luctions were frequency-specific, occurring only in
OAEs elicited when f_2 was within the noise exposure
id. This level of noise (for this particular noise spec-
m) probably approximates the minimum level of contin-
us noise required to induce a threshold shift in guinea
gs. In addition, the DPOAE amplitude alterations
monstrated here lend support to the conclusions of Chen
al. (1995) that chronic low-level noise exposure induces
olecular changes in the OHCs which may, in turn, alter
chlear function.

cknowledgements

The authors wish to thank David Owens for writing the
mputer programs used to run our signal processing
uipment and Sharon G. Kujawa, Ph.D., for intellectual
d technical help during the initial stages of this work.
pported by DAMD 17-93-V-3013, T32-DC0007, NIH
ant R01-DC00722, Kam's Fund for Hearing Research
d the Louisiana Lions Eye Foundation.

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Conditioning the auditory system with continuous vs. interrupted noise of equal acoustic energy: Is either exposure more protective?

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Abstract

The purpose of this study was to test the hypothesis that differences exist in the amount of protection provided by prior sound conditioning with continuous vs. interrupted, moderate-level noise. Differences were determined by monitoring the changes that occurred in cubic ($2f_1$-f_2) distortion product otoacoustic emission (DPOAE) amplitude growth functions subsequent to a traumatizing noise exposure (105 dB SPL, 1.0-2.0 kHz octave band noise presented 24 hours per day for 3 days) in guinea pigs which had been conditioned with either continuous (89 dB SPL, 1.0-2.0 kHz octave band noise presented 24 hours per day for 11 days) or interrupted noise (95 dB SPL, 1.0-2.0 kHz octave band noise presented on a 6 hours "on"/18 hours "off" schedule for 11 days) of equal acoustic energy.

Results suggest that there are significant differences in the degree of protection provided by prior sound conditioning with the continuous and interrupted schedules of moderate-level noise used in this study. Specifically, the interrupted conditioning protocol afforded some degree of protection against the damaging effects of the traumatizing noise exposure, limited to frequencies above the noise exposure band. Conversely, there was a lack of any consistent and sizable protective effect found across the entire test frequency range for the continuous sound conditioning protocol.

Keywords: Distortion product otoacoustic emissions, Protection, Noise exposure

1. Introduction

In recent years, considerable attention has been given to the idea that susceptibility of the auditory system to noise-induced hearing loss may be lessened by an individual's previous history of noise exposure. Prior exposures to moderate-level acoustic stimulation (i.e., conditioning) can reduce the deleterious effects of subsequent higher level (and usually damaging) exposures (Canlon et al., 1988, 1992; Campo et al., 1991; Henderson et al., 1992; Ryan et al., 1994). Both continuous and interrupted schedules of moderate-level noise have been used as conditioning exposures, and both schedules have been reported as being effective in providing protection against subsequent noise trauma.

There is evidence to suggest that the temporal pattern of a noise exposure (i.e., continuous vs. interrupted presentation) is an important factor in determining the magnitude of hair cell damage and the pattern of sensitivity change produced by an exposure. Many investigations have focused on the histological changes associated with continuous and interrupted exposure schedules and have shown that continuous noise exposures are more damaging to the cochlea than interrupted exposures of equal acoustic energy (Bohne et al., 1985, 1987; Fredelius and Wersäll, 1992). Others have reported that moderate-level continuous and interrupted noise exposures differ in the pattern of auditory sensitivity change that they produce over time (Carder and Miller, 1972; Miller et al., 1963). Continuous noise exposure results in a decrease in sensitivity that grows over the first 24 hours of exposure and then stabilizes as the exposure continues. This pattern is referred to as an asymptotic threshold shift (Carder and Miller, 1972). Interrupted noise exposure, on the other hand, results in an initial decrease in sensitivity during the first few days of exposure, followed by a

return towards pre-exposure levels on subsequent days of exposure (Clark et al., 1987; Miller et al., 1963). This pattern of change is known as "toughening".

Currently, it is unknown whether there are differences in the amount of protection afforded by prior conditioning of the auditory system with either moderate-level continuous or interrupted noise exposures of equal acoustic energy. Thus, the purpose of this study is to test the hypothesis that differences exist in the amount of protection provided by prior sound conditioning with continuous vs. interrupted moderate-level noise. The differences in protective effect of the two conditioning schedules were determined by monitoring changes that occurred in distortion product otoacoustic emission (DPOAE) amplitude growth functions after a subsequent traumatizing exposure in guinea pigs that had been conditioned. Both conditioning exposure schedules had the same total acoustic energy. This equal energy requirement was considered important in the design of the study because it yielded noise exposures that differed only in their temporal pattern.

2. Methods

2.1. Subjects

Experiments were performed on pigmented guinea pigs (*Cavia cobaya*) of either sex weighing between 500 and 850 grams. Only animals with no obvious outer or middle ear pathology were included in the study. An additional criterion was also established to exclude animals whose DPOAE responses may have been altered during surgery. This criterion required that all animals have DPOAE amplitudes of 10 dB or greater when f_1=10 kHz, f_2=12 kHz (DPOAE frequency = 8 kHz), and the level of the primary tones was 45 dB SPL. These

primary frequencies were thought to be outside of the range affected by the octave band noise (1-2 kHz) used in this study. Therefore, reductions in amplitude below the 10 dB criterion level were associated with surgical complications. Animals were randomly assigned to six groups (n = 14 per group) as follows:

Aged Normal Group - Animals were housed and cared for in the Animal Care facility for 7-8 weeks prior to being tested. The level of the ambient noise within this facility was usually between 40 and 50 dB SPL (A-scale).

Continuous Conditioning Group - Animals were exposed continuously for 11 days to an 89 dB SPL (A-scale) octave band noise (1-2 kHz). These animals were tested within 2 hours after their removal from the noise exposure booth.

Interrupted Conditioning Group - Animals were exposed to a 95 dB SPL (A-scale) octave band noise (1-2 kHz) 6 hours per day for 11 days (6 hrs "on"/18 hrs "off"). These animals were also tested within 2 hours after their removal from the noise exposure booth.

Continuous Conditioning, then Blast Group - Animals were exposed continuously for 11 days to an 89 dB SPL octave band noise (1-2 kHz), given 1 week to recover, exposed continuously to a 105 dB SPL noise (1-2 kHz) for 3 days, and then allowed to recover for 4 weeks before being tested.

Interrupted Conditioning, then Blast Group - Animals were exposed 6 hours per day for 11 days to a 95 dB SPL octave band noise (1-2 kHz; 6 hours "on"/18 hours "off"), given 1 week to recover, exposed continuously to a 105 dB SPL noise (1-2 kHz) for 3 days, and then allowed to recover for 4 weeks before being tested.

Blast Only Group - Animals were exposed only to the 105 dB SPL noise (1-2 kHz)

for 3 days, and then allowed to recover for 4 weeks before being tested. The amount of time these animals spent in the facility prior to exposure was equivalent to the time required to condition the animals in the other groups (11 days) and allow them to recover (1 week).

During periods of noise exposure, the animals were housed in a small sound attenuating booth (See Section 2.2.1 for description). Unexposed animals, along with animals recovering from the noise exposure, were housed and cared for in the Louisiana State University Medical Center's Animal Care facility. All animals were given free access to food and water. The care and use of the animals were approved by the Medical Center's Institutional Animal Care and Use Committee.

2.2 Noise Generation and Exposure Methods

2.2.1 Noise exposure facility

During periods of noise exposure, unanesthetized guinea pigs were housed in groups of 8 or less in a small sound-attenuated booth (approximate interior dimensions 76 × 60 × 40 cm). A speaker was mounted on a wooden surface which covered the booth ceiling and was approximately 40 cm above the level of the guinea pigs' ears. The walls of the booth were lined with hard, reflective surfaces to produce uniform sound levels throughout the chamber. A light was installed within the booth and controlled with a timer to provide the animals with 12 hours of light and 12 hours of darkness.

2.2.2 Noise generation and calibration procedures

Both the moderate-level conditioning noise and the traumatizing noise were generated

by a WG2 Waveform Generator (Tucker-Davis Technologies) which was set in the "Uniform" mode. This signal was bandpass filtered using a Brickwall Filter (Wavetek/Rockland Model 753A) with a low frequency cutoff at 1.0 kHz, a high frequency cutoff at 2.0 kHz, and a roll-off of 115 dB/octave to yield an octave band of noise centered at 1414 Hz. The level of the filtered noise was controlled by a PA4 Programmable Attenuator (Tucker-Davis Technologies). Additional power was gained using a power amplifier (McIntosh MC2100) producing the final signal that was delivered to the speaker (Realistic 40-1286C; 8Ω, 30 watts).

The continuous and interrupted schedules of the moderate-level conditioning noise had equal acoustic energy, i.e., the intensity levels of the continuous and interrupted noise were set to provide equal energy per 24 hours of exposure. Thus, the continuous conditioning noise was presented at a level of 89 dB SPL (A-scale) for 24 hours per day for 11 days, while the interrupted conditioning noise was presented at a level of 95 dB SPL (A-scale) for 6 hours per day (6 hours "on"/18 hours "off") for 11 days. The high-level traumatizing noise was presented continuously for 3 days at 105 dB SPL (A-scale). These levels were chosen because they approximated the levels of conditioning and traumatizing noises used in previous studies concerned with this type of protection phenomenon (Canlon et al., 1988; Campo et al., 1991).

Noise levels were monitored twice a week using a ½ inch condenser microphone (Brüel & Kjaer Type 4133) and preamplifier combination that were connected to a measuring amplifier (Brüel & Kjaer Type 2610). This system was calibrated with a sound level calibrator (Brüel & Kjaer Type 4230). The microphone was positioned so that it was

approximately at the level of the guinea pigs' ears and was placed at various positions around the booth to ensure that the noise was equally distributed throughout the booth. Noise levels were found to vary ± 2 dB within the booth depending upon microphone position. The background level inside the booth with the noise off, doors closed, and animals present was approximately 40 dB SPL (A-scale). The voltage across the speaker was checked daily using a digital voltmeter (Wavetek Corporation BI-DM15XL). The voltages corresponding to the levels of noise used in this study, i.e., 89, 95, and 105 dB SPL, were approximately 0.45, 0.90, and 2.9 volts (rms).

2.3 General surgical methods

Immediately prior to testing, the animals were anesthetized by administering a dose of urethane (ethyl carbamate, Sigma; 1.5 g/kg, i.p.), tracheotomized, and allowed to breathe unassisted. Supplementary doses of anesthetic (urethane, 0.15 g/kg) were given if necessary to maintain an adequate depth of anesthesia (as indicated by a lack of a withdrawal response to deep pressure and pain applied to the animal's paw). Electrocardiogram and rectal temperature were monitored throughout each experiment and temperature was maintained at 38 ± 1°C using a heating pad.

The surgical procedures used were similar to those described by Kujawa et al. (1993). Using a ventrolateral approach, the right auditory bulla was exposed and opened to gain access to the tendons of the middle ear muscles. These tendons were sectioned in all animals to prevent the involvement of middle ear muscle contraction on the DPOAE measurements. The surgical procedures required approximately 1½ hours to complete.

2.4 DPOAE generation and calibration procedures

The instrumentation used in this study to elicit and measure DPOAEs has been previously described (Skellett et al., 1996). Briefly, primary tones, f_1 and f_2 ($f_2>f_1$), were generated under computer control using Tucker-Davis System 2 audio processing equipment and then sent to separate speakers (Etymotic Research, ER-2) housed within an acoustic probe assembly. The acoustic probe assembly was tightly coupled directly to the right ear of each animal. DPOAEs were detected by a sensitive microphone (Etymotic Research, ER-10) also housed within the probe assembly and amplified using a microphone preamplifier (Etymotic Research, ER-1072). A dynamic signal analyzer (Hewlett-Packard, 3561A) was used to average the DPOAE responses for fast Fourier transform analysis and spectral display (25 rms averages; center frequency = DPOAE frequency; span = 1 kHz; bandwidth = 3.75 Hz).

Calibration of the primary tones was performed at the outset of the study and then twice a week thereafter. This was accomplished by coupling the acoustic probe assembly to a ¼ inch condenser microphone (Brüel & Kjaer Type 4135) and then cross-checking the output of the speakers using: (1) the ER-10 probe microphone; and (2) the B&K condenser microphone. The signal from the probe microphone and preamplifier was sent to the signal analyzer and the level of each of the primary tones was obtained from the spectral display (center frequency = primary tone frequency; span = 1 kHz; bandwidth = 3.75 Hz) and later converted to dB SPL (re: 20 μPa). The signal transduced by the condenser microphone and preamplifier was sent to the measuring amplifier and the level of the primary tones was indicated by the deflection of the needle on the meter scale (measured in dB SPL). The equivalence of the primary levels using both measurement systems was verified at each

calibration session.

2.5 DPOAE amplitude growth function measurements

Cubic ($2f_1$-f_2) DPOAEs were measured for several combinations of f_1 and f_2. The particular values of f_2 (707, 1000, 1414, 2000, 2828, 4000, 5656, 8000, and 11,312 Hz) were chosen for study because their corresponding frequencies map out in half octave steps the frequency spectrum of the noise band used to expose the animals and the region of possible OHC loss along the cochlear partition. The f_2/f_1 ratio was held constant at 1.2, which is within the range describing the optimal frequency separation of f_1 and f_2 for the guinea pig (Brown, 1987; Brown and Gaskill, 1990). DPOAE responses were elicited with equilevel primary tones which were presented in descending order, starting at a level of 70 dB SPL and decreasing in 5 dB steps to 20 dB SPL. The amplitudes of the DPOAEs, defined as the spectral peak corresponding to the $2f_1$-f_2 frequency, were recorded manually in dBV from the FFT spectra (25 averages) and later converted to dB SPL (re: 20 μPa). Plots of primary level vs. DPOAE amplitude (amplitude growth functions) were generated.

2.6 Data management and analysis

Statistical analysis of the data was performed using SigmaStat® Statistical Software (Version 2.0 for Windows® 95, NT, & 3.1; Jandel Scientific Corporation). The response measurements (DPOAE amplitude growth functions) of all exposure groups were analyzed using a between-group, three-way (exposure group x frequency x primary level) analysis of variance (ANOVA) procedure. The Tukey multiple comparisons (post hoc) test was

performed when significant differences were found. P values less than 0.05 were considered statistically significant.

3. Results

3.1 Baseline DPOAE responses

The mean DPOAE amplitude growth functions obtained from the Aged Normal Group (Figs. 1 and 3) were consistent with the normative DPOAE results of other studies that used guinea pigs as experimental subjects (Brown, 1987; Brown and Gaskill, 1990). The DPOAE amplitudes were generally 30-50 dB SPL below the level of the primary tones at each of the intensity levels and each frequency tested. Although there were instances where the amplitude growth functions were nonmonotonic (e.g., f_2 = 707 and 1000 Hz), the overall growth rate of the distortion product amplitudes was approximately linear. The variability of the amplitude growth functions across animals was small at each frequency tested, with standard deviations of usually less than 3 dB.

3.2 Effects of sound conditioning on DPOAE responses

The DPOAE amplitude growth functions shown in Fig. 1 represent the average responses (mean ± S.E.; n=14) of the Continuous Conditioning Group and the Interrupted Conditioning Group. These are plotted in contrast with the average responses (mean ± S.E.; n=14) of the Aged Normal Group to demonstrate the effect of both sound conditioning protocols on normal DPOAE responses and to compare the effects of the two different schedules of conditioning noise. All main effects (Exposure Group, Frequency, Intensity) and

interactions of the statistical analysis were statistically significant ($P<0.001$). The Group x Frequency interaction was not significant when the intensity of the primary tones was 20 dB ($P = 1.000$); however, this interaction was significant at all other intensity levels. In addition, the Group x Intensity interactions were not significant when the frequency of f_2 was 8000 ($P = 0.826$) and 11312 Hz ($P = 0.997$), whereas significant interactions were found at all other f_2 frequencies.

3.2.1 Continuous sound conditioning

The continuous conditioning exposure caused reductions in DPOAE amplitudes in a frequency- and intensity-dependent manner. The overall bell shape of Fig. 2a demonstrates the frequency-dependence of the noise-induced effects on the DPOAE responses. The maximum effect of the noise exposure occurred in the mid-frequency range of the frequencies tested, while effects were not as great in the lower and higher frequencies. Specifically, the greatest amplitude reductions (approximately 12-18 dB) occurred in the frequency range spanning f_2 frequencies of 1414-4000 Hz. Smaller reductions (approximately 5-8 dB) were found at f_2 frequencies of 707, 1000, and 5656 Hz, while at 8000 and 11312 Hz, the magnitude of the amplitude reductions was only 1-3 dB.

The pattern of the individual bars plotted at each frequency in Fig. 2a illustrates the intensity-dependence of the noise-induced effects on the DPOAE responses. In most cases, the greatest decreases in amplitude occurred for primary levels ranging from 45-60 dB SPL. At the lower f_2 frequencies (707 and 1000 Hz), there were actually places within the amplitude growth functions where the responses of the sound conditioned group were better

or not much different than those of the normal control group. This occurred for primary levels ranging from 50-60 dB SPL. It is within this intensity range where a large dip occurred in the amplitude growth functions of the unexposed group of animals.

3.2.2 Interrupted sound conditioning

The interrupted conditioning exposure also caused reductions in DPOAE amplitudes in a frequency- and intensity-dependent manner. The overall "quasi" bell-shaped pattern of Fig. 2b demonstrates the frequency-dependence of the noise-induced effects on the DPOAE responses. The maximum effect of the noise exposure occurred in the mid-frequency range of the frequencies tested, while effects were not as great in the lower and virtually non-existent in the higher frequencies. For this exposure protocol however, the greatest amplitude reductions occurred in a narrower frequency range (relative to the continuous sound conditioning protocol), spanning f_2 frequencies of 2000-4000 Hz. The magnitude of the reductions within this range was approximately 11-16 dB.

The pattern of the individual bars plotted at each frequency in Fig. 2b illustrates the intensity-dependence of the noise-induced effects on the DPOAE responses. In the frequency region where the interrupted noise exposure had its greatest effect (f_2 = 2000-4000 Hz), the largest decreases in amplitude occurred for primary levels ranging from about 40-60 dB SPL. At the lower f_2 frequencies (707 and 1000 Hz), there were actually intensity levels of the amplitude growth functions where the DPOAEs amplitudes recorded for the sound conditioned group were larger than those of the normal control group. These elevations in the DPOAE amplitudes relative to the normal controls occurred for primary levels ranging from

50-60 dB SPL. It is within this intensity range where a large dip occurred in the amplitude growth functions of the unexposed group of animals.

3.3 Effects of the traumatizing noise exposure on the DPOAE responses of unconditioned and sound conditioned animals

The DPOAE amplitude growth functions shown in Fig. 3 represent the average responses (mean ± S.E.; n=14) of the Blast Only Group, the Continuous Conditioning then Blast Group, and the Interrupted Conditioning then Blast Group. These are plotted along with the average responses (mean ± S.E.; n=14) of the Aged Normal Group to demonstrate the effects of the traumatizing noise exposure in both unconditioned and sound conditioned animals. When comparing the effects of the traumatizing noise exposure on the DPOAE amplitude growth function measured within the unconditioned vs. sound conditioned groups, statistically significant differences were found between the exposure groups ($p=0.015$). Results show that overall (data pooled over frequency and intensity), the Interrupted Conditioning then Blast was least affected by the traumatizing noise, followed closely by the Blast Only Group and then the Continuous Conditioning then Blast Group. This implies that there was some amount of protection afforded by prior sound conditioning with the interrupted moderate-level noise exposure used in this study. However, the overall effect of the continuous sound conditioning protocol seemed to render the auditory system more susceptible to the traumatizing noise.

3.3.1 Continuous sound conditioning

Fig. 4a provides a graphical representation of the mean amplitude differences between

the Blast Only Group and the Continuous Conditioning then Blast Group. The amplitude differences between the two groups, for the most part, were fairly small, with maximum differences of 1-3 dB (on average). In addition, no obvious pattern of protection was observed when comparing the DPOAE amplitudes of these two groups. This means that there were no definite frequency regions that demonstrated that the continuous conditioning noise was either helpful or harmful in protecting against the subsequent traumatic noise exposure.

3.3.2 Interrupted sound conditioning

Fig. 4b provides a graphical representation of mean amplitude differences between the Blast Only Group and the Interrupted Conditioning then Blast Group. Interrupted sound conditioning produced a dual effect on the DPOAE responses depending upon the f_2 test frequency. The results revealed a trend toward an increased susceptibility to the traumatizing noise exposure in the sound conditioned animals in the lower test frequency range (f_2 = 707-2000 Hz) and a degree of protection in the test frequency range spanning f_2 frequencies of 2828-11312 Hz. The differences between the groups were statistically significant at f_2 frequencies of 707, 1000, 8000, and 11312 Hz ($P<0.05$).

4. Discussion

4.1 Effects of sound conditioning on DPOAE responses

4.1.1 Continuous sound conditioning

The maximum effect of the continuous noise exposure occurred at test frequencies within and slightly above the noise exposure band, with lesser effects outside of this

frequency region. Specifically, DPOAE amplitude reductions of 12-18 dB occurred in the frequency region spanning f_2 frequencies of 1414-4000 Hz. This frequency region corresponds to the center and upper cutoff frequency of the noise exposure band used in this study (1.0-2.0 kHz), and extends to one octave above the upper cutoff frequency. This finding is consistent with previous reports showing that the maximum effect of an exposure can occur in the same frequency region of the exposure and/or approximately ½-1 octave above the upper cutoff frequency for bands of noise of reasonably constant spectrum level (Davis et al., 1943; Ward, 1976; Mitchell et al., 1977; Salvi et al., 1982; Ryan et al., 1994; Fowler et al., 1995).

However, the results of the present study were different from the findings of Canlon and Fransson (1995), who also tested the effects of continuous sound conditioning on DPOAEs in guinea pigs. These authors reported that their continuous sound conditioning protocol did not cause any significant alterations in $2f_1$-f_2 DPOAE amplitudes. A possible explanation might be that DPOAE frequencies chosen for study by Canlon and Fransson (1995) were not generated within the region along the cochlear partition that was affected by their pure tone conditioning exposure. Again, the region of DPOAE generation is thought to be near, or at, the f_2 place on the cochlear partition (Matthews and Molnar, 1986; Brown et al., 1992; Allen and Fahey, 1993; Puel et al., 1995). This means that if the exposure was such that it caused only very localized changes in cochlear function in the region of the conditioning tone, then DPOAEs with primary frequencies (especially f_2) outside of this region would be, for the most part, unaltered by the exposure. Recently, Skellett et al. (1996) showed that reductions in DPOAE amplitudes in response to a low-level exposure occurred

only when the frequency of f_2 was within the noise exposure band.

4.1.2 Interrupted sound conditioning

The maximum effect of the interrupted noise exposure occurred in the mid-frequency range of the frequencies tested, while effects were not as great in the lower, and virtually non-existent in the higher frequencies. For this exposure protocol however, the greatest amplitude reductions occurred in a narrower frequency range relative to the continuous sound conditioning protocol, spanning f_2 frequencies of 2000-4000 Hz. This frequency region corresponds to the upper cutoff frequency of the noise exposure band and extends to one octave above the upper cutoff frequency. Subramaniam et al. (1995) also found that by the final day of their high-frequency interrupted exposure, reductions in DPOAE amplitudes were fairly localized to frequencies within the noise exposure band. However, Subramaniam et al. (1994a,b) found that when they exposed animals to a low-frequency noise, substantial reductions in DPOAE amplitudes were measured even at high frequencies. This spread of cochlear damage in response to low frequency noise exposure has been previously observed (Zurek et al., 1982; Clark et al., 1987; Subramaniam et al., 1991).

At the lower f_2 frequencies (707 and 1000 Hz), there were intensity levels where the DPOAEs amplitudes recorded for the sound conditioned group were larger than those of the normal control group. The elevations in the DPOAE amplitudes relative to the normal controls occurred for primary levels ranging from 50-60 dB SPL. Elevated DPOAE amplitudes measured in sound conditioned animals at frequencies bordering the noise exposure band have also been reported by Boettcher and Schmiedt (1995). In addition, Kim

et al. (1992) found that DPOAE amplitudes were larger than normal at the edge of a hearing loss in subjects who exhibited audiograms that were typical of those obtained in subjects with prior histories of noise exposure.

4.1.3 Comparison of the effects of the continuous and interrupted sound conditioning protocols

Although the two sound conditioning protocols used in the present experiment had equal acoustic energy, the resultant changes in DPOAE amplitude growth functions measured in the groups of guinea pigs exposed to either continuous or interrupted noise were not equivalent. Not only were the magnitudes of the noise-induced DPOAE amplitude reductions less in the animals conditioned with the interrupted noise as opposed to the continuous noise, but the range of f_2 frequencies affected by the interrupted exposure was also decreased. These results do not support the validity of the EEH which assumes that the cumulative damage to the auditory system is a function of the total acoustic energy received, regardless of the distribution of energy over time (Eldred et al., 1955). In addition, previous histological data has shown that while the pattern of hair cell damage was the same for a continuous vs. an interrupted noise exposure (both with equal acoustic energy), the magnitude of hair cell damage induced by the exposures was less for the interrupted exposure (Bohne et al., 1985, 1987; Fredelius and Wersäll, 1992). More recently, Chang and Norton (1996) reported that an intermittent noise exposure caused less reductions in DPOAE amplitudes than a continuous noise exposure, a finding similar to that found in the present study. Thus, it is quite possible that the quiet periods within the interrupted exposure acted to minimize noise-induced hair cell damage and were responsible for the significantly smaller reductions in the DPOAE

amplitudes (when compared to the continuous exposure) observed in this study.

4.2 Did prior sound conditioning provide protection against the damaging effects of the traumatizing noise exposure?

4.2.1 Continuous sound conditioning

The overall effect of the continuous sound conditioning protocol seemed to render the auditory system more susceptible to the traumatizing noise exposure. The lack of protection demonstrated here could possibly be related to the fact that the moderate-level continuous conditioning exposure chosen for study may have, by itself, been enough to cause irreversible damage to the outer hair cells, thus precluding the possibility of providing protection against the damaging effects of the subsequent traumatizing exposure. It has been suggested that protection against subsequent noise trauma may be less likely to occur in the presence of a threshold shift induced by the sound conditioning exposure (Canlon et al, 1992; Ryan et al., 1994; Canlon and Fransson, 1995). However, when a group of sound conditioned animals was tested after a one week rest period, DPOAE responses were comparable to those found in normal animals (data not shown).

4.2.2 Interrupted sound conditioning

Sound conditioning with the interrupted protocol prior to exposure to the traumatic noise exposure produced a dual effect on the DPOAE responses depending upon the f_2 test frequency. The results revealed a trend towards an increased susceptibility to the traumatizing noise exposure in the sound conditioned animals in the lower test frequency range (f_2 = 707-2000 Hz) and some degree of protection in the test frequency range spanning

f_2 frequencies of 2828-11312 Hz. This pattern of protection has not been previously reported. The frequency-related pattern of protection reported by other investigators usually shows that there is significant protection afforded by prior sound conditioning with an interrupted protocol within the frequency region of the exposure band which extends to higher frequencies (Campo et al., 1991; Henderson et al., 1992; Subramaniam et al., 1992, 1993). The lack of protection demonstrated in this study for the lower frequency region (coinciding with the noise exposure band) could possibly be related to the fact that the moderate-level interrupted conditioning noise used may have, by itself, caused irreversible damage to the outer hair cells within this frequency region. Permanent damage to the cells within this region of the cochlea may have precluded the protective role of the sound conditioning exposure, and rendered the auditory system more susceptible to the deleterious effects of subsequent noise trauma. However, as for the continuous conditioning protocol, DPOAE responses were comparable to those found in normal animals when measurements were taken after a one week rest period (data not shown).

4.2.3 Comparison of the effects of the traumatizing noise exposure on the DPOAE responses of animals conditioned with continuous vs. interrupted moderate-level noise – Was either sound conditioning protocol more protective?

The primary purpose of this study was to test the hypothesis that differences exist in the amount of protection provided by prior sound conditioning with continuous versus interrupted moderate-level noise of equal acoustic energy. The results suggest that overall, there were significant differences in the degree of protection provided by prior sound conditioning with continuous vs. interrupted schedules of moderate-level noise of equal

acoustic energy. It appears that interrupted sound conditioning provided more protection against the damaging effects of the subsequent traumatic exposure than continuous sound conditioning. While no definite frequency regions were identified demonstrating that continuous sound conditioning was either helpful or harmful in protecting against the subsequent traumatic noise exposure, the effectiveness of the interrupted sound conditioning protocol in providing protection was highly frequency-dependent. In the frequency range encompassing the noise exposure band and extending down to ½ octave below the lower cutoff frequency, neither sound conditioning exposure was effective in providing protection against the subsequent traumatizing exposure. However, when the frequencies of f_2 generating the DPOAEs were limited to the frequency region above that of the noise exposure band, the results indicate that some degree of protection was afforded by the interrupted sound conditioning protocol. Although there have been other attempts to compare the effectiveness of the two different schedules of moderate-level conditioning noise (Fowler et al., 1995; White et al., 1996), neither demonstrated any degree of protection against subsequent traumatic exposure.

5. Summary and Conclusions

Both continuous and interrupted schedules of moderate-level noise have been shown to be effective in providing protection against the damaging effects of subsequent noise trauma. The results of the present study again support the protective role of an interrupted sound conditioning exposure protocol. However, unlike the findings of other studies using similar conditioning protocols (Campo et al., 1991; Henderson et al., 1992; Subramaniam et al.,

1993), the effectiveness of the interrupted moderate-level noise exposure in providing protection was highly frequency-dependent, limited only to the frequency region above that of the noise exposure band. In addition, while other investigators have demonstrated the protective role of continuous sound conditioning (Canlon et al., 1988, 1992; Ryan et al., 1994; Canlon and Fransson, 1995), the results of this study do not support their findings.

6. Acknowledgements

This research was completed in partial fulfillment of the first author's dissertation requirements for the degree of Doctor of Philosophy in The Department of Communication Sciences and Disorders at Louisiana State University, Baton Rouge, LA. The authors thank M. Jane Collins, Ph.D., Denny LeCompte, Ph.D., Janna Oetting, Ph.D., and George Strain, Ph.D. for their expert advice. The authors also wish to thank David and Steven Owens for writing the computer programs used to control the signal processing equipment and for the electrical engineering support they provided. This work was supported by NIH Grants (P01-DC00379, R01-DC00722, and T32-DC0007), DAMD 17-93-V-3013, AASERT grant #DAAH04-94-G-0111, Kam's Fund for Hearing Research and the Louisiana Lions Eye Foundation.

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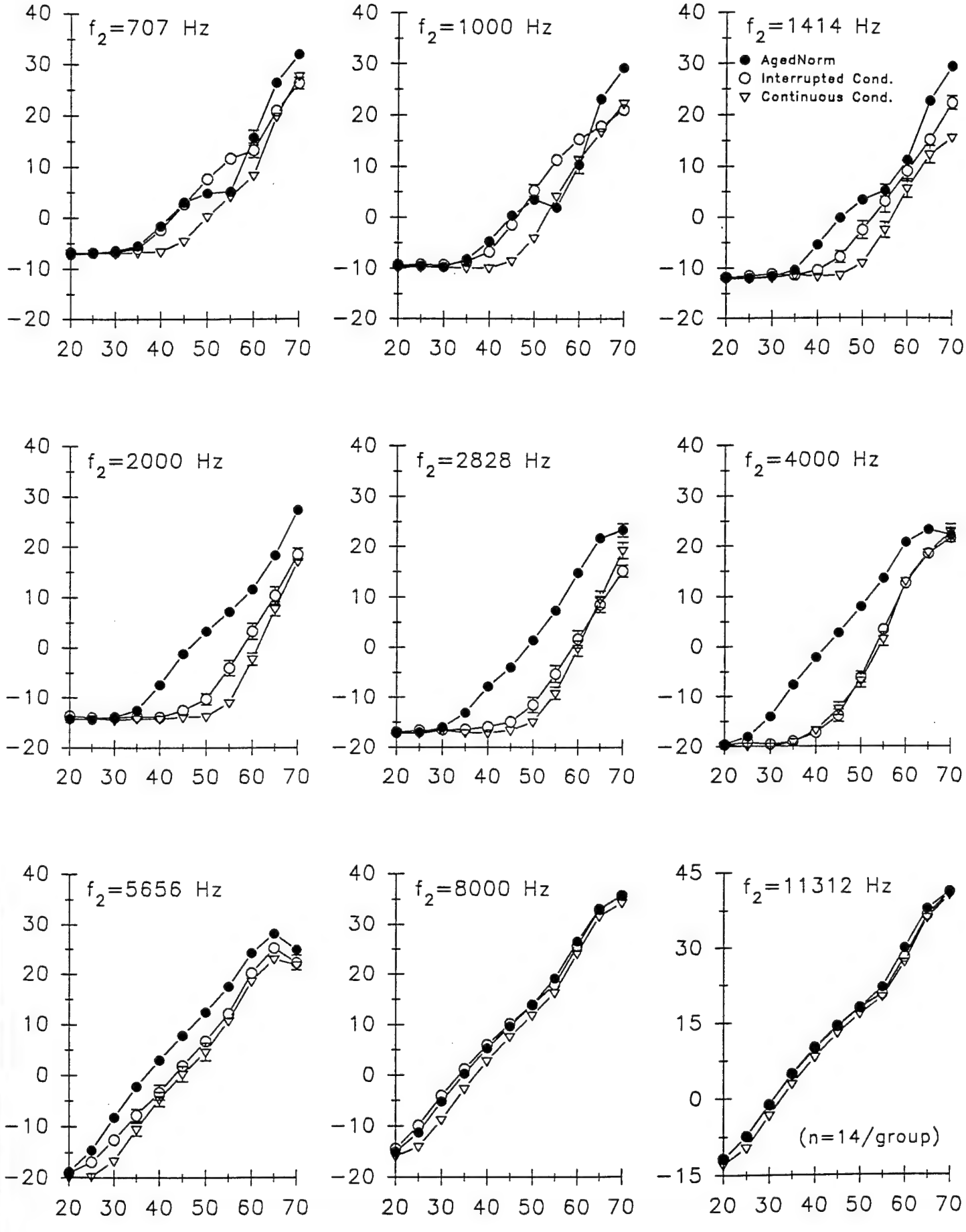
Fig. 1. The effect of sound conditioning on DPOAE responses. DPOAE amplitude growth functions obtained from the Continuous Conditioning Group and the Interrupted Conditioning Group are plotted along with the functions of the Aged Normal Group for f_2 frequencies of 707, 1000, 1414, 2000, 2828, 4000, 5656, 8000, and 11,312 Hz. Data are presented as mean DPOAE amplitude ± S.E. as a function of primary level (20-70 dB SPL).

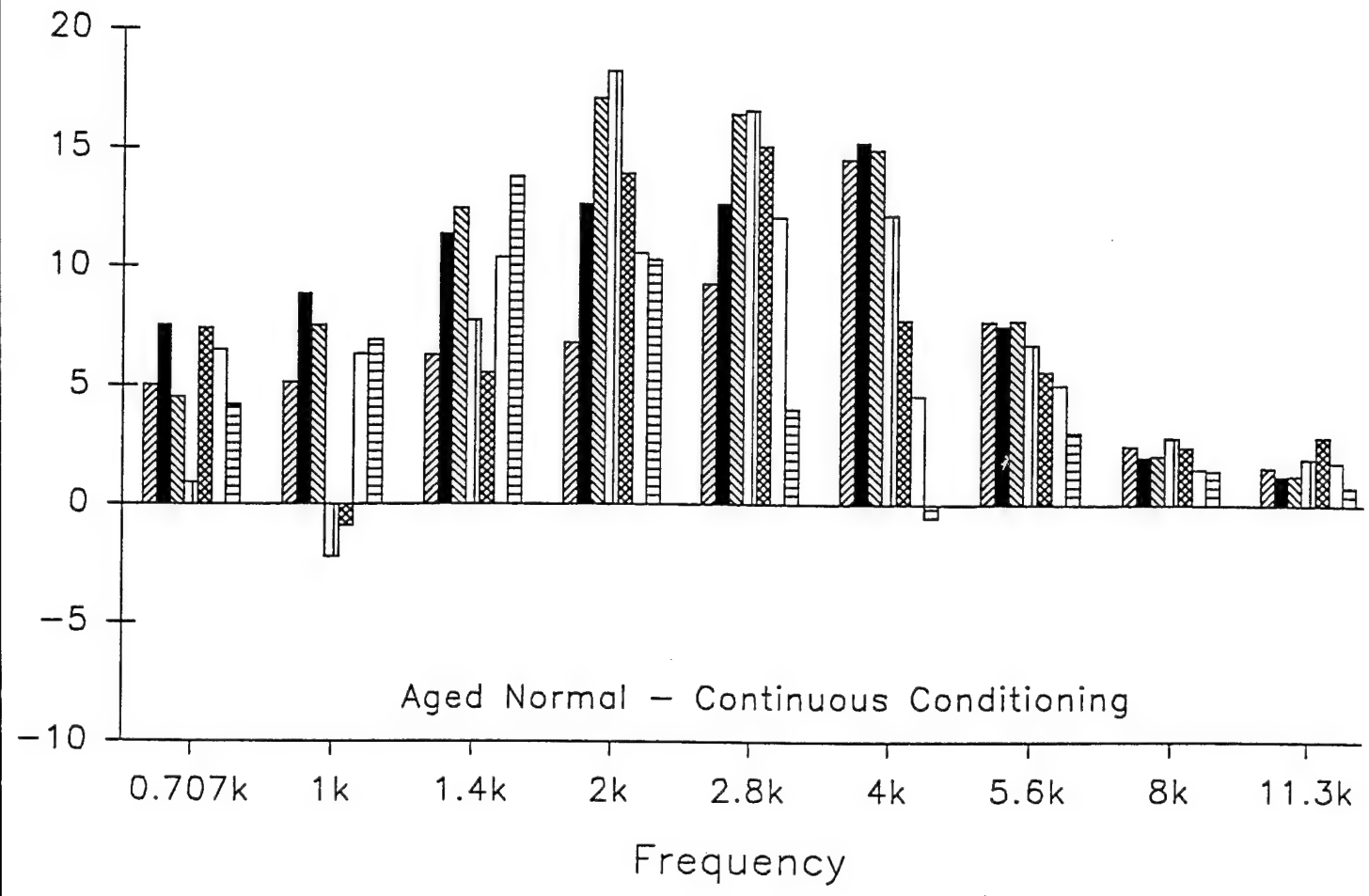
Fig. 2. Comparison of the effects of the continuous and interrupted conditioning exposures on normal DPOAE responses. Data were derived by subtracting the mean DPOAE amplitudes of (top) the Continuous Conditioning Group and (bottom) the Interrupted Conditioning Group (bottom) from the mean DPOAE amplitudes of the Aged Normal Group (from Fig. 1). Data are presented as the mean amplitude difference (in dB) plotted as a function of f_2 frequency (707-11,312 Hz) for several different primary intensity levels (40-70 dB SPL; inset). Positive excursions from zero represent the magnitude of the DPOAE amplitude reductions induced by the conditioning exposures, whereas negative excursions show where the magnitude of the responses were actually larger in the noise exposed groups than in the normal group.

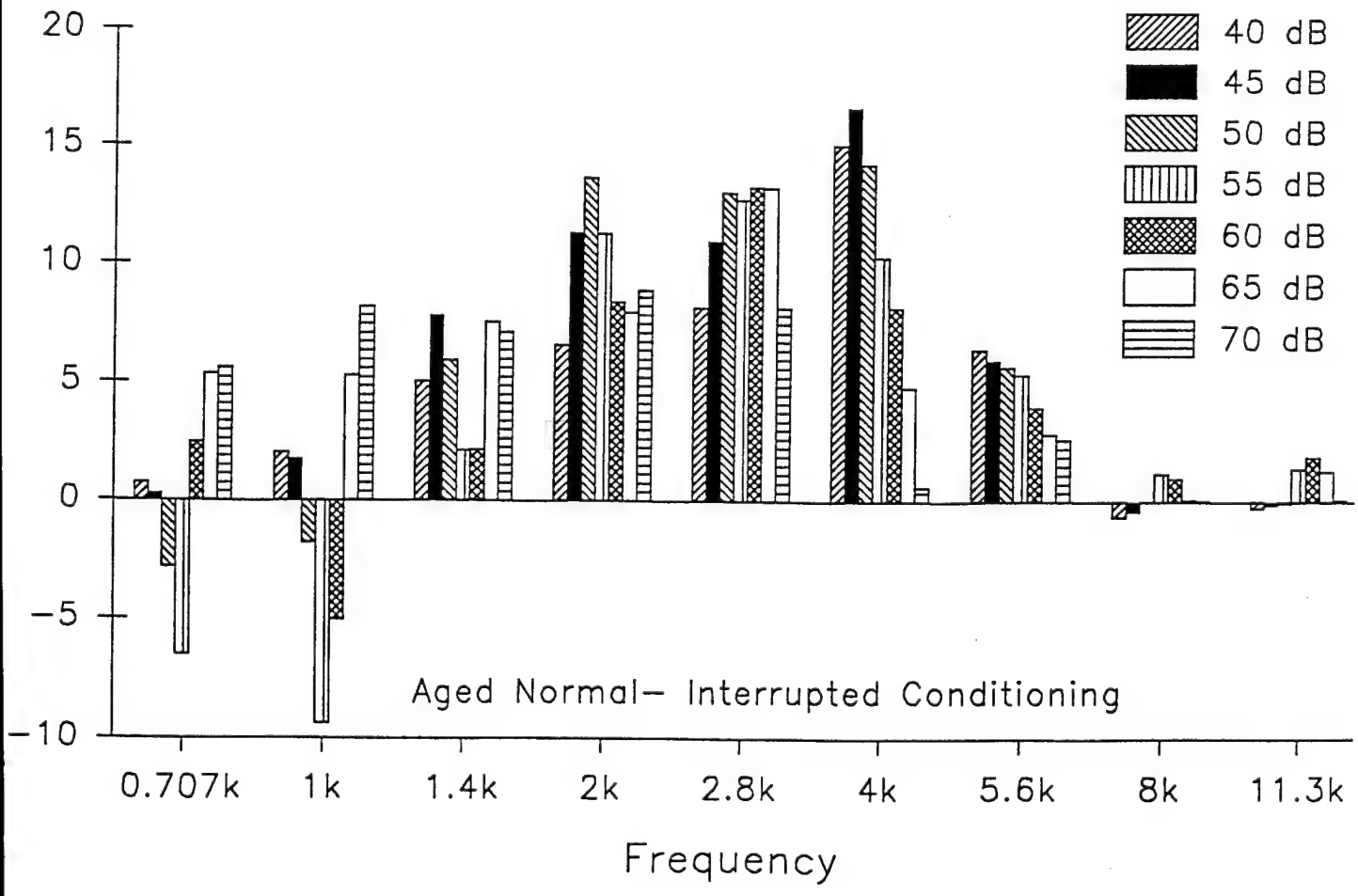
Fig. 3. The effect of the traumatizing noise exposure on DPOAE responses of unconditioned and sound conditioned animals. DPOAE amplitude growth functions obtained from the Blast Only Group, the Continuous Conditioning then Blast Group, and the Interrupted Conditioning then Blast Group are plotted along with the functions of the Aged Normal Group for f_2 frequencies of 707, 1000, 1414, 2000, 2828, 4000, 5656, 8000, and 11,312 Hz. Data are

presented as mean DPOAE amplitude ± S.E. as a function of primary level (20-70 dB SPL).

Fig. 4. Comparison of the effects of the traumatizing noise exposure on the DPOAE responses of unconditioned vs. sound conditioned animals. Data were derived by subtracting the mean DPOAE amplitudes of (top) the Continuous Conditioning then Blast Group and (bottom) the Interrupted Conditioning then Blast Group from the mean DPOAE amplitudes of the Blast Only Group (from Fig. 3). Data are presented as the mean amplitude difference (in dB) plotted as a function of f_2 frequency (707-11,312 Hz) for several different primary intensity levels (40-70 dB SPL; inset). Positive excursions from zero represent where sound conditioning acted to enhance the DPOAE amplitude reductions of the traumatic noise exposure. Negative excursions from zero show where the magnitudes of the DPOAE responses were actually larger in the sound conditioned groups than in the unconditioned group (i.e., protection).


f2=707 Hz
f2=1000 Hz
f2=1414 Hz
AgedNorm
Interrupted Cond.
Continuous Cond.
f2=2000 Hz
f2=2828 Hz
f2=4000 Hz
f2=5656 Hz
f2=8000 Hz
f2=11312 Hz
(n=14/group)
Primary Level (dB SPL)


20
15
10
5
0
−5
−10
Aged Normal – Continuous Conditioning
0.707k
1k
1.4k
2k
2.8k
4k
5.6k
8k
11.3k
Frequency


20
15
10
5
0
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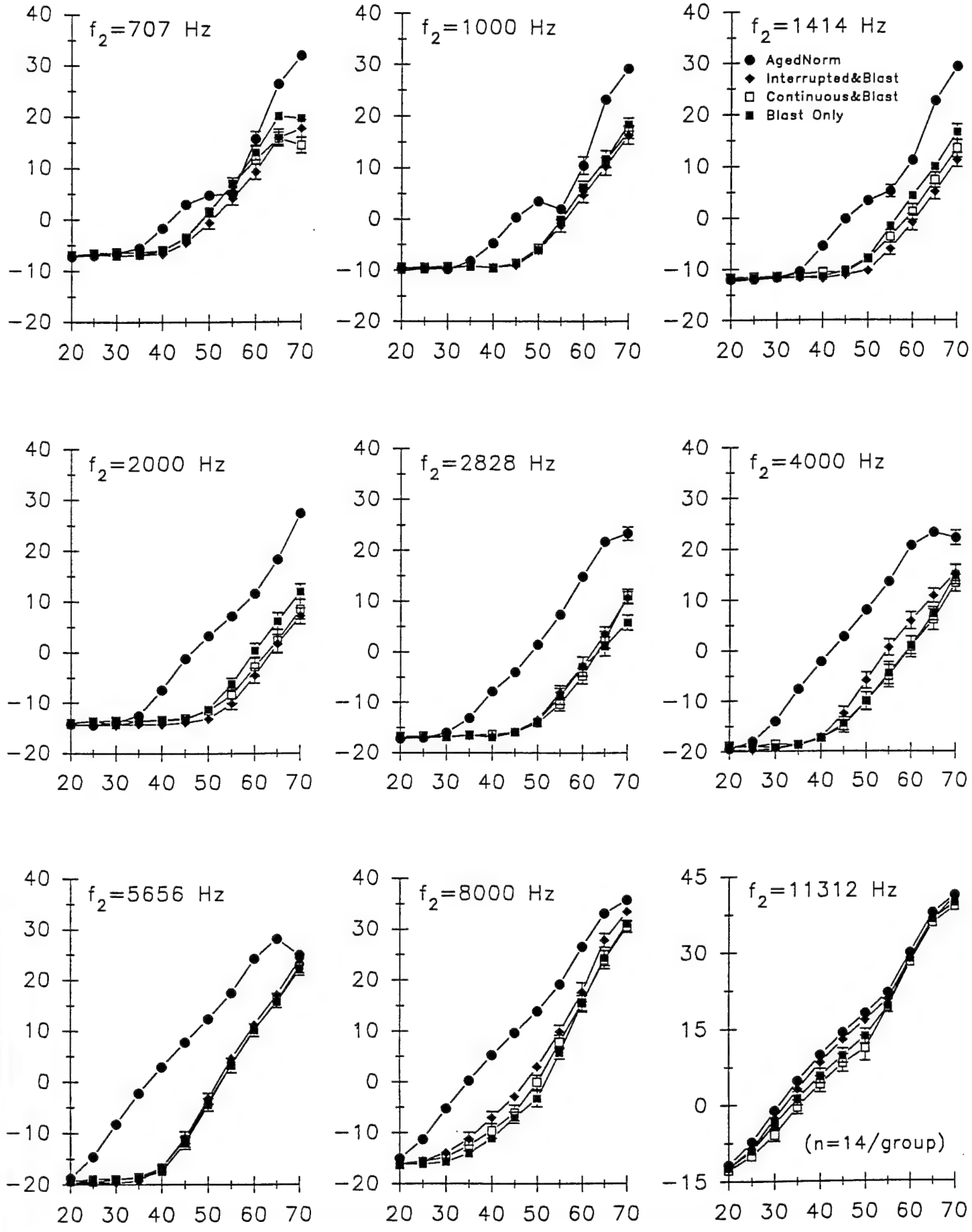
−10
40 dB
45 dB
50 dB
55 dB
60 dB
65 dB
70 dB
Aged Normal– Interrupted Conditioning
0.707k
1k
1.4k
2k
2.8k
4k
5.6k
8k
11.3k
Frequency


f2=707 Hz
f2=1000 Hz
f2=1414 Hz
AgedNorm
Interrupted&Blast
Continuous&Blast
Blast Only
f2=2000 Hz
f2=2828 Hz
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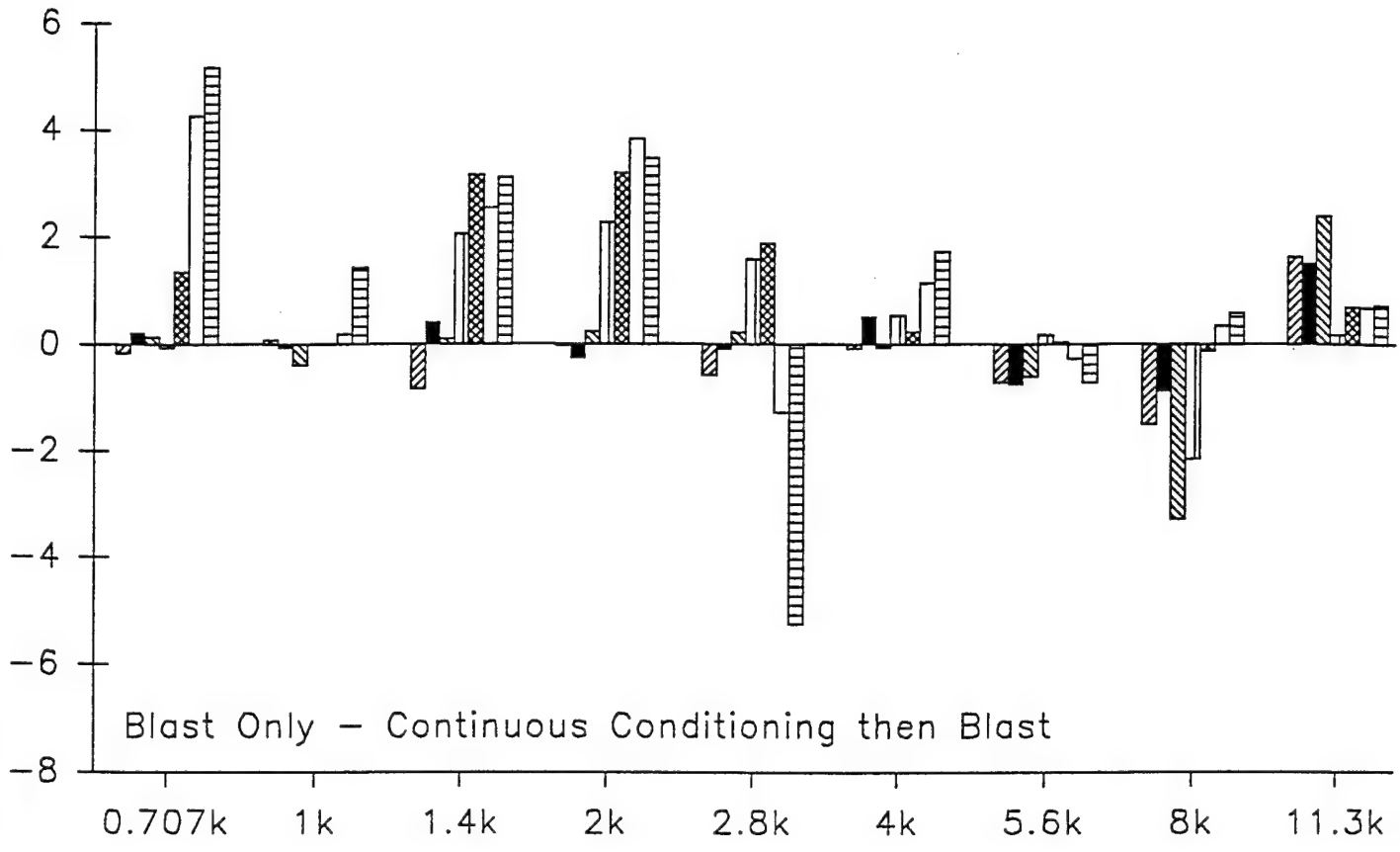
f2=8000 Hz
f2=11312 Hz
(n=14/group)
Primary Level (dB SPL)


6
4
2
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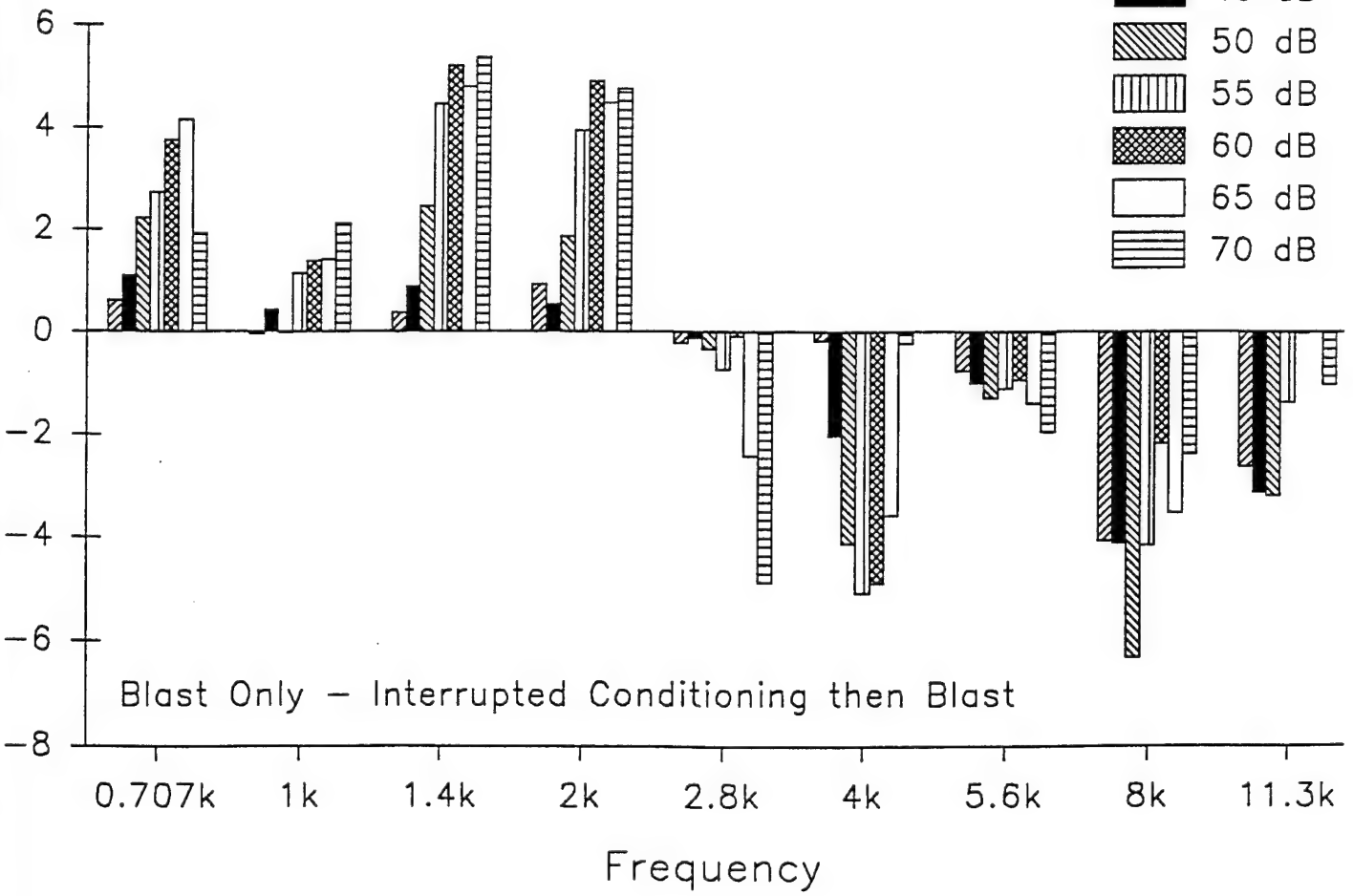
−2
−4
−6
−8
Blast Only − Continuous Conditioning then Blast
0.707k
1k
1.4k
2k
2.8k
4k
5.6k
8k
11.3k
Frequency


40 dB
45 dB
50 dB
55 dB
60 dB
65 dB
70 dB
6
4
2
0
−2
−4
−6
−8
Blast Only − Interrupted Conditioning then Blast
0.707k
1k
1.4k
2k
2.8k
4k
5.6k
8k
11.3k
Frequency

Appendix #7
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Hearing Research 110 (1997) 87–94

HEARING RESEARCH

Differences in the distribution of responses to ATP and acetylcholine between outer hair cells of rat and guinea pig

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Received 16 December 1996; revised 10 April 1997; accepted 16 April 1997

Abstract

Adenosine 5′ triphosphate (ATP) and acetylcholine (ACh) are neurotransmitters (ACh) and/or modulators (ATP) in the mammalian cochlea. In guinea pig, it appears that both neurotransmitters have a similar response distribution, with larger responses being evoked by the ligands in short hair cells compared to long hair cells (e.g., Chen et al., 1995b. Noise exposure alters the response of outer hair cells to ATP. *Hear. Res.* 88, 215–221.; Erostegui et al., 1994. In vitro pharmacologic characterization of a cholinergic receptor on outer hair cells. *Hear. Res.* 74, 135–147). The purpose of the present study was to test whether the distribution of responses to ACh and ATP in the OHCs of rat is the same as guinea pig. The ligand-induced current was monitored using the whole-cell configuration of the patch-clamp technique. Results show that in guinea pig OHCs, extracellular application of 100 μM ATP induced a current response in a majority of the same cells that responded to the application of 100 μM ACh. In contrast in rat OHCs, 100 μM ATP did *not* induce a current in the majority of cells that responded to the application of 100 μM ACh. *N*-methyl-glucamine (NMG^+) substituted for K^+ in the pipette solution failed to unmask an ATP-evoked inward current in rat OHCs. In addition, no response was produced in rat or guinea pig OHCs by adenosine, adenosine 5′-monophosphate (AMP) or adenosine 5′-diphosphate (ADP) at 100 μM. Results suggest that in guinea pig ACh-gated channels are present on most of the same OHCs that have ATP-gated ion channels, whereas in rat ACh-gated ion channels are present without ATP-gated channels on some OHCs.

Keywords: Receptor; Cochlea; Ion channel; Voltage-clamp

1. Introduction

Adenosine 5′-triphosphate (ATP) has been suggested to subserve both neurotransmitter and neuromodulator functions in a number of neuronal systems (see review: Burnstock, 1990). At least five types of ATP receptors have been identified based on physiological and pharmacological characteristics (Kennedy and Leff, 1995). Both ligand gated (ionotropic) and G protein coupled (metabotropic) ATP receptors have been cloned (Lustig et al., 1993; Webb et al., 1993; Brake et al., 1994; Filtz et al., 1994; Valera et al., 1994; Housley et al., 1995a,b; Surprenant et al., 1995; Collo et al., 1996).

Increasing evidence has shown that ATP may act as a neurotransmitter or neuromodulator in the mammalian cochlea (Bobbin, 1996; Eybalin, 1993). In vivo experiments of Bobbin and Thompson (1978) and Kujawa et al. (1994a) show that ATP and related agonists (e.g., ATP-γ-S) placed in the perilymph compartment have significant effects on the compound action potential of the auditory nerve (CAP), cochlear microphonics (CM), summating potential (SP), as well as distortion product otoacoustic emissions (DPOAEs) in guinea pigs. In subsequent studies, it was demonstrated that the ATP antagonists, cibacron blue, basilen blue and suramin, exert profound influences on sound-evoked responses, suggesting that endogenous release of ATP affects cochlear function via the P_2 subtype of purinergic receptors (Kujawa et al., 1994b).

In vitro studies indicate that ATP activates recep-

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PII S0378-5955(97)00069-5

tors on several cells in the cochlea. ATP activates an ionotropic receptor on isolated outer hair cells (OHCs) that induces inward cation currents (Na^+, Ca^{2+}) and depolarizes the cell membrane (Ashmore and Ohmori, 1990; Chen et al., 1995a,b; Housley et al., 1992; Ikeda et al., 1991; Kakehata et al., 1993; Kujawa et al., 1994b; Nakagawa et al., 1990; Nilles et al., 1994; Shigemoto and Ohmori, 1990). ATP also activates a metabotropic receptor that increases intracellular free Ca^{2+} in these cells (Ashmore and Ohmori, 1990; Ikeda et al., 1991; Shigemoto and Ohmori, 1990). Additional evidence for a metabotropic receptor mechanism was obtained by Niedzielski and Schacht (1992) which showed an increase in ionositol phosphate accumulation in guinea pig organ of Corti following incubation with ATP agonists (see also Ogawa and Schacht, 1993, 1994, 1995). A heterogeneous modulation of L-type Ca^{2+} channel currents by ATP in OHCs of guinea pig cochlea was reported (Chen et al., 1995a). Activation of ATP receptors increases intracellular Ca^{2+} levels in IHCs (Dulon et al., 1991), Deiters' cells (Dulon et al., 1993) and Hensen's cells (Dulon et al., 1993) and depolarizes both Deiters' cells (Dulon, 1995) and inner hair cells (IHCs; Dulon et al., 1991). Wangemann (1996) demonstrated Ca^{2+}-induced release of ATP from whole organ of Corti. Other evidence demonstrates that ATP induces changes in the cells of the stria vascularis (Lui et al., 1995; Suzuki et al., 1995; Wangemann, 1995), and the origin of the endogenous ATP that acts on these receptors may be the marginal cells in the stria vascularis (White et al., 1995). ATP occurs in high concentrations in endolymph (Munoz et al., 1995a), and when placed in the endolymph, ATP induces large changes in cochlear function (Munoz et al., 1995b). Thus ATP appears to have an important paracrine and/or autocrine role in the cells of the cochlea.

In the guinea pig, it appears that the neurotransmitters, ATP and acetylcholine (ACh), both have a similar response distribution, with larger responses being evoked by the ligands in short hair cells compared to long hair cells (e.g., Chen et al., 1995b; Erostegui et al., 1994; Housley et al., 1995b). This distribution of the ACh evoked response is in harmony with the anatomical data indicating a decreasing density of cholinergic efferent terminals on OHCs from the basal turn of the cochlea towards the apical turn in guinea pig and rat (Altschuler and Fex, 1986; Eybalin, 1993). Therefore, the purpose of the present study was to determine if the distribution of responses to ATP and ACh in OHCs of rat is the same as guinea pig. The ligand induced current was monitored using the whole-cell configuration of the patch-clamp technique. Preliminary results have been presented (Chen et al., 1996b).

2. Materials and methods

2.1. Isolation of OHCs and Deiters' cells

OHCs and Deiters' cells from 37 pigmented guinea pigs, 56 Sprague Dawley rats and 9 pigmented Long Evans rats were isolated as described previously (Zajic and Schacht, 1987; Chen et al., 1995a,b). Unless stated otherwise, the data presented from rat are from the Sprague Dawley strain. Animals were anesthetized with pentobarbital (35 mg/kg, i.p. for guinea pigs and 50 mg/kg, i.p. for rats), decapitated, and the bulla separated and placed in a modified Hank's balanced saline (HBS). The bone surrounding the cochlea was removed, and the organ of Corti was placed in 200 μl of HBS containing collagenase (1 mg/ml, Type IV, Sigma) for 5 min. The cells were then transferred into the dishes containing a 150 μl drop of HBS using a microsyringe and stored at room temperature. The length of each cell was measured with a calibrated reticulum prior to recording.

2.2. Whole-cell voltage clamp

Single dispersed rat or guinea pig OHCs and Deiters' cells were voltage or current clamped using the whole-cell variant of the patch-clamp technique (Hamill et al., 1981) with an Axopatch-1D patch-clamp amplifier (Axon Instruments). Patch electrodes were fabricated from borosilicated capillary tubing (Longreach Scientific Resources) using a micropipette puller (Sutter Instrument Co.), and fire polished on a microforge (Narashige Scientific Instrument Lab.) prior to use. Membrane currents were filtered at 5 KHz (−3dB) using a four-pole low-pass Bessel filter digitized with a 12-bit A/D converter (DMA Interface, Axon Instruments), and stored for off-line analysis using a pentium microcomputer. Voltage paradigms were generated from a 12-bit D/A converter (DMA Interface, Axon Instruments) using pClamp software (Axon Instruments). After establishment of the whole-cell configuration, series resistance and cell capacitance compensation were carried out prior to recording with 80% series resistance compensation normally applied. As reported previously (Chen et al., 1996a), for rat and guinea pig OHCs whole-cell capacitance was about 20–25 pF and series resistance was about 5.4 MΩ. No subtraction of leakage current was made.

2.3. Solutions

The HBS utilized for isolating cells and perfusing the bath contained (mM): 145 NaCl, 5.4 KCl, 2.5 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES and 10 glucose. The HBS solution was adjusted to a pH of 7.40 with NaOH and to 300 mOsm/kg H_2O with sucrose. The K^+ internal so-

lution (solution filling the patch pipettes) contained (mM): 140 KCl, 0.5 $MgCl_2$, 5 HEPES, 11 EGTA, 0.1 $CaCl_2$, 2 Na_2ATP and 0.1 Na_2GTP. The *N*-methyl-glucamine (NMG^+) internal solution contained (mM): 120 NMG^+, 35 TEA-Cl, 11 EGTA, 0.5 $CaCl_2$, 10 HEPES, 4 MgATP, 0.1 Na_2GTP and 5 sucrose. Both internal solutions were adjusted to a pH of 7.35 with HCl and had an osmolality of 284 mOsm/kg H_2O which was adjusted with sucrose. The ATP and its related agonists tested were prepared daily at desired concentrations in the HBS external solution. The drugs used were: ATP, adenosine 5′-0-(3-thiotriphosphate) tetralithium salt (ATP-γ-S), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), adenosine (Adeno) and acetylcholine (ACh). All the drugs were purchased from Sigma Chemical Company (St. Louis, MO). All the drug solutions were delivered from a U-tubing system as described previously (Erostegui et al., 1994; Chen et al., 1995a,b). All experiments were conducted at room temperature (22 ~ 24°C). The care and use of the animals reported on in this study were approved by the Animal Care and Use Committees of Louisiana State University Medical Center.

3. Results

3.1. Effects of ATP on rat and guinea pig OHCs

Our laboratory previously reported (Chen et al., 1996a) that the average length of the rat OHCs studied were shorter (mean, 32 μm) than those from guinea pig (mean, 48 μm), and that there was no significant difference in the resting membrane potential of the rat OHCs (mean, −66 mV) compared to guinea pig (about −69 mV).

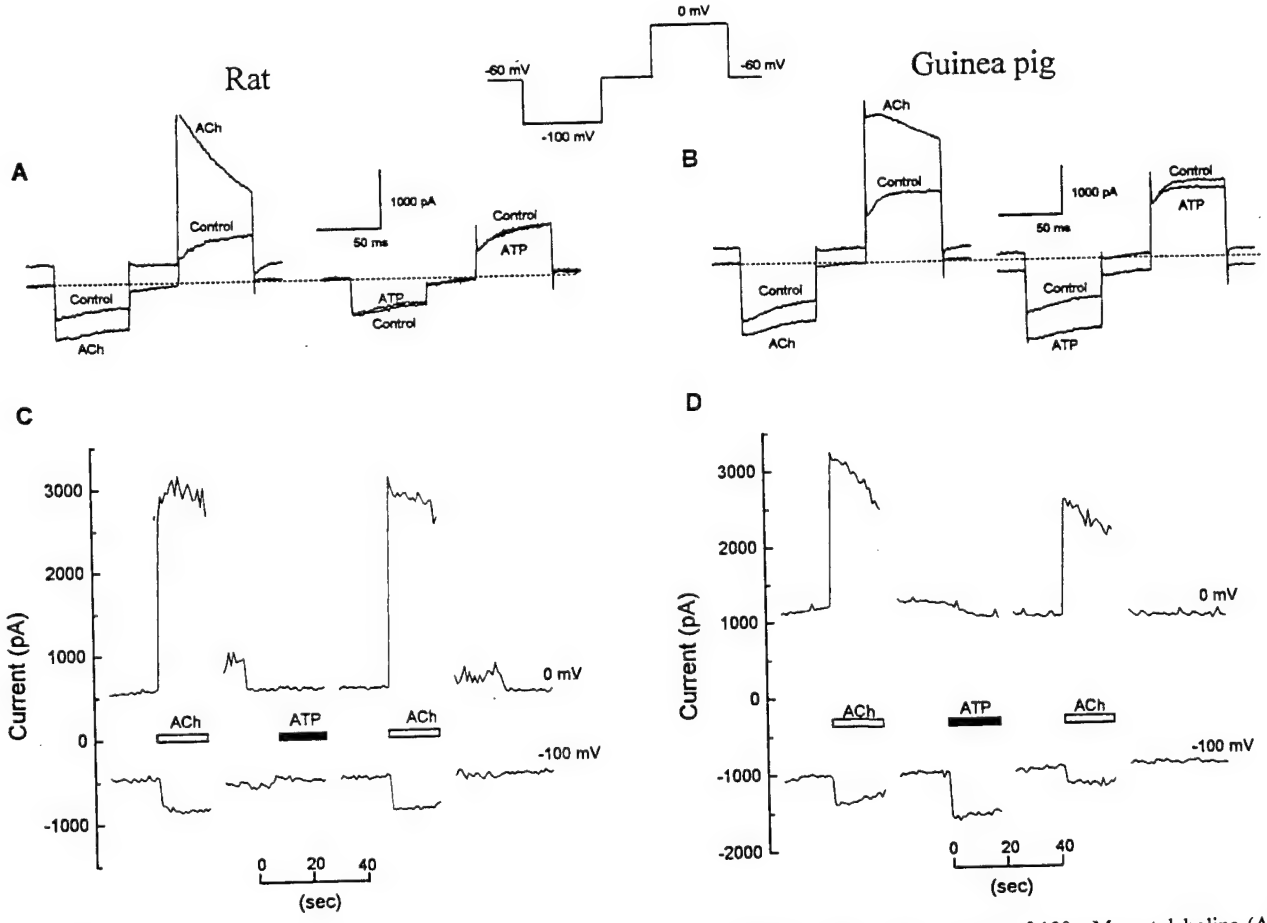

Fig. 1. A: Superimposed step-evoked currents recorded from a rat OHC in the absence (control) and presence of 100 μM acetylcholine (ACh), and recorded from the same cell in the absence (control) and presence of 100 μM ATP. B: Superimposed step-evoked currents recorded from a guinea pig OHC in the absence (control) and presence of 100 μM ACh, and recorded from the same cell in the absence (control) and presence of 100 μM ATP. In both A and B the dashed line is the zero current level. C: Time course of the ACh and ATP-induced responses at −100 mV and 0 mV in a rat OHC. D: Time course of the ACh and ATP-induced responses at −100 mV and 0 mV in a guinea pig OHC. Traces were constructed utilizing the protocol shown for A and B from the current value measured 25ms after the onset of the hyperpolaring step to −100 mV and 5ms after the onset of the depolarizing step to 0 mV from a holding potential of −60 mV with steps presented every second. Drug was applied for 20 step presentations. Recovery but not wash-out is shown.

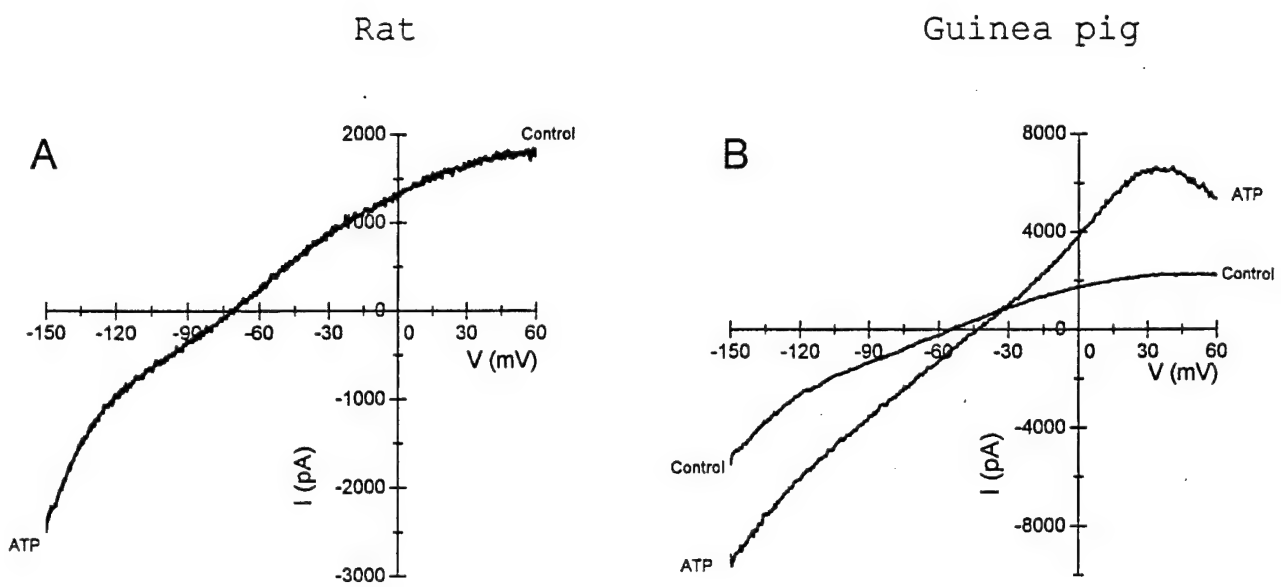

Fig. 2. A: Current–voltage (*I–V*) relationships recorded from a rat outer hair cell (OHC) in the absence and presence of 100 μM ATP. B: *I–V* curves recorded from a guinea pig OHC in the absence and presence of 100 μM ATP. The voltage command was ramped from −150 mV to +60 mV (0.5 mV/ms). Membrane potential was held at −60 mV.

In guinea pigs, very long OHCs were not studied, since they do not respond to either ACh or ATP with large currents (Chen et al., 1995a; Erostegui et al., 1994). In contrast, all rat OHCs that met criteria (cylindrical shape, no swelling, nucleus at the base of the cell, no Brownian motion of the organelles) were studied. Both ACh and ATP at 100 μM, a concentration which is near the maximum on their respective dose response curves, were applied to the majority of OHCs isolated from rat and guinea pig. Fig. 1 shows that 100 μM ACh evoked an inward current at −100 mV and an outward current at 0 mV in OHCs of both rat ($n=64$ out of 67 cells tested) and guinea pig ($n=56$, out of 58 tested). In contrast, 100 μM ATP ($n=40$) evoked a current response in only two out of 40 of the rat OHCs tested that responded to ACh (33 μM to 1 mM; $n=40$), but ATP (100 μM) evoked a current response in 42 of the 45 guinea pig OHCs responding to ACh (100 μM). In three additional rat OHCs 1 mM ATP was tested and gave no response though the cells responded to ACh (100 μM). The two rat OHCs that responded to ATP were shorter than average (20 and 25 μm), however, 5 additional cells of the same length did not respond to ATP. Fig. 2 illustrates the lack of effect of ATP on the current–voltage (*I–V*) relationship in OHCs from rat (Fig. 2A) and the large increase in inward current induced by ATP in OHCs from the guinea pig as reported previously (Fig. 2B; Chen et al., 1995a,b). The large outward current obtained in response to the application of ATP in Fig. 2B is related to the time ATP is in contact with the cell as demonstrated by the slowly developing ATP-induced outward current shown in Fig. 3 (guinea pig). A possible explanation is that an ATP-induced increase in internal Ca^{2+} subsequently activates a Ca^{2+} dependent outward K^+ current.

3.2. Effects of ion substitution

The ATP-induced current is a cation current that is usually directed inward at potentials negative to 0 mV (Chen et al., 1995b; Nakagawa et al., 1990). The current is carried by Na^+ and Ca^{2+} (Nakagawa et al., 1990). There is the possibility that a Ca^{2+} activated, outward K^+ current masks the ATP-evoked inward current in rat OHCs. Therefore, to prevent an outward K^+ current, NMG^+ was substituted for K^+ in the pipette. However, even under these conditions, ATP (100 μM) failed to induce a detectable inward current in rat OHCs ($n=4$).

3.3. Effects of ATP analogues

It is possible that the ionotropic ATP receptor on rat OHCs may have a different pharmacology than the receptor on guinea pig OHCs. Therefore, we tested the effects of ADP, AMP and adenosine. As shown in Fig. 3, at a concentration of 100 μM these agents did not induce any detectable current response in OHCs from either guinea pig or rat (guinea pig OHCs: ADP, $n=9$; AMP, $n=7$; adenosine, $n=12$; rat OHCs: ADP, $n=8$; AMP, $n=6$; adenosine, $n=6$). Due to the fact that ATP breakdown is relatively fast, we employed a more slowly hydrolyzable ATP analog, ATP-γ-S, to test whether there is an ATP-induced current in rat OHCs. As shown in Fig. 4, extracellular application 100 μM ATP-γ-S did not induce a detectable current response in OHCs isolated from rat ($n=6$),

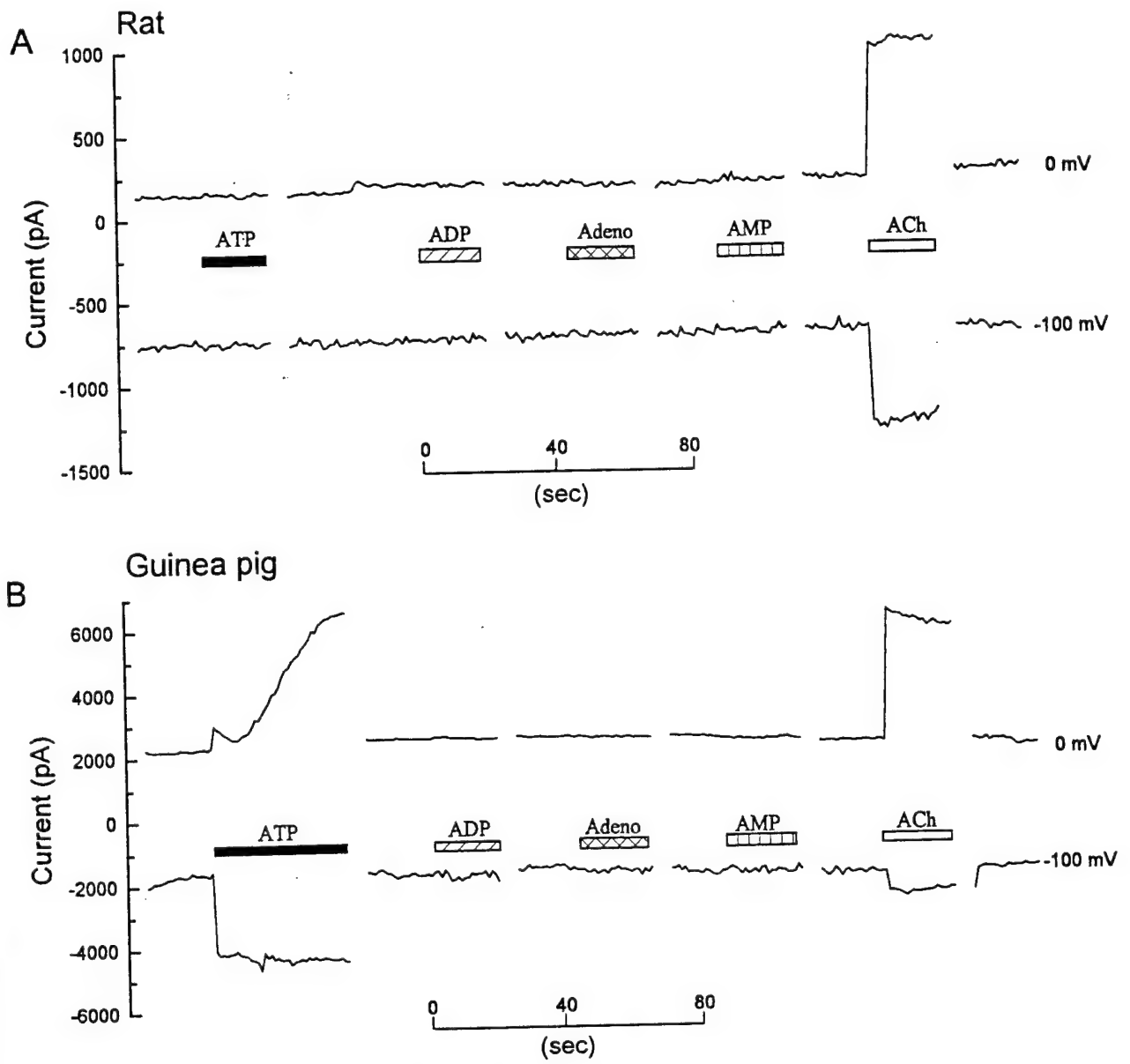

Fig. 3. Examples of the current responses of a rat OHC (A) and a guinea pig OHC (B) to the application of adenosine, AMP, ADP, ATP and ACh. Voltage protocol and trace construction was the same as in Fig. 2. Drug application is indicated by the bars and all drug concentrations were100 μM.

but did elicit an inward current in OHCs ($n=4$) isolated from guinea pig (Fig. 3D).

3.4. Effects of ATP on Deiters' cells

Another explanation for ATP not inducing a detectable current in a larger proportion of rat OHCs is that the isolation procedure was generally destructive to ligand receptors. However, ATP (10 μM) evoked a large inward current that was rapidly desensitized in Deiters' cells isolated from the cochleae of both rat ($n=7$) and guinea pig ($n=7$; Fig. 5). In vivo, Deiters' cells are attached to the OHCs and, although their ionotropic ATP receptors may be slightly different, one would expect that any degradation induced by the isolation procedure would affect both receptors to the same extent.

3.5. Effects of pigmentation or strain

To test for a relationship between the ATP-evoked current response and the degree of pigment in the rat or strain of rat, OHCs were obtained from 9 pigmented rats (Long Evans). ATP (100 μM) did not evoke a detectable current in nine of the OHCs tested, but seven of these cells did demonstrate a typical current change in response to the application of 100 μM ACh. Two cells did not respond to either ATP or ACh.

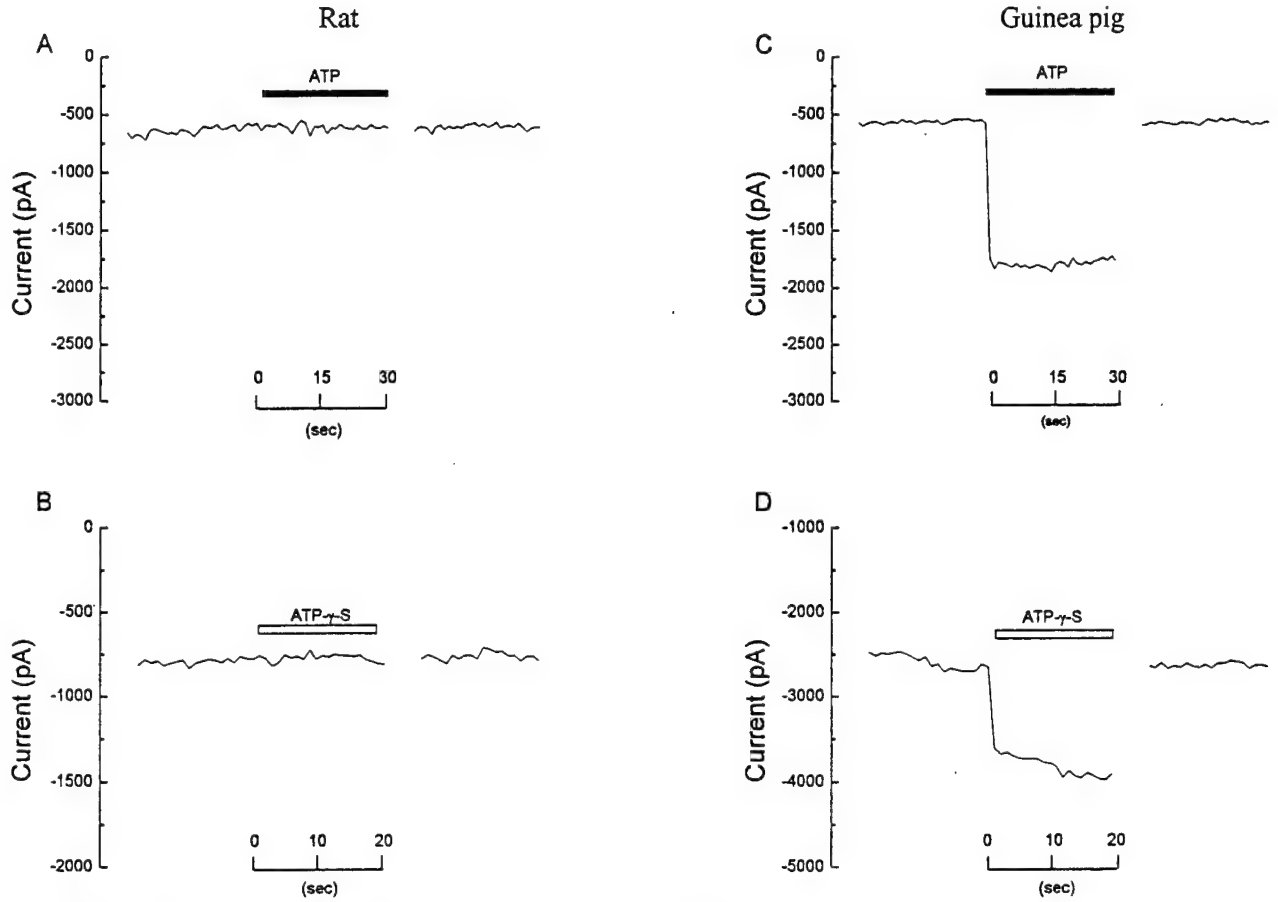

Fig. 4. Example of the absence of a detectable current response from a rat OHC at −100 mV during extracellular application of 100 μM ATP (A) or from another rat OHC to 100 μM ATP-γ-S (B). Examples of guinea pig OHCs elicited a current response at −100 mV during extracellular application of 100 μM ATP (C) or from another OHC 100 μM ATP-γ-S (D). Current was elecited by a 70 ms hyperpolaring step to −100 mV from a holding potential of −60 mV with steps presented every second. Drug was applied for 20 or 30 step presentations and recovery but not wash-out is shown.

4. Discussion

Anatomical data indicates a decreasing density of cholinergic efferent terminals on OHCs from the basal turn of the cochlea (short OHCs) towards the apical turn (long OHCs) in both rat and guinea pig (Altschuler and Fex, 1986; Eybalin, 1993). In addition, it appears that both ACh and ATP have a similar response distribution in the guinea pig, with larger responses being evoked by the ligands in short hair cells compared to long hair cells (Chen et al., 1995b; Erostegui et al., 1994; Housley et al., 1995b). This was confirmed in the present study in that guinea pig OHCs which responded to ACh also responded to ATP. In contrast, in the present study, the majority of rat OHCs that responded to ACh failed to respond to ATP with an inward current. This may indicate that the ATP and ACh response distribution in the rat is different from the guinea pig. The rat may not be unusual in its lack of response to ATP since similar observations have been made in chick short hair cells. As shown here in rat OHC, chick short hair cells which respond to ACh do not respond to ATP (McNiven et al., 1996).

One must be circumspect in attributing the lack of ATP-induced response to the lack of ionotropic receptors, since the absence of a response in a particular cell may be due to physical or chemical damage to the ATP receptors. Investigators have suggested that the ionotropic receptor on OHCs is located near the stereocilia portion of the cells (Housley et al., 1992; Mockett et al., 1994, 1995). Stereocilia are delicate structures and are easily damaged during the physical isolation of the OHCs. Thus the lack of response to ATP may be due to some physical alteration in the receptors located near or on damaged stereocilia. Yet, this does not seem to be the case with guinea pig OHCs, which appear to exhibit ATP-induced currents even in OHCs with damaged stereocilia (unpublished observations). Alternatively, the receptor may have been damaged by collagenase or another chemical released from the tissue during the isolation procedure. However, this seems unlikely given the large inward currents evoked by ATP in Deiters' cells isolated from the same rat cochlea, indicating

that ATP receptors in Deiters' cells were not chemically altered into a non-functioning state.

What the results may indicate is a functional difference between rat OHCs, chick short hair cells, and guinea pig OHCs. At present, one can only speculate about this possibility. For example, Chen et al. (1995b) observed that exposing guinea pigs to low levels (64 dB SPL) of chronic (10 days) noise modified the magnitude of the ATP-induced current observed in OHCs, increasing it in long OHCs and decreasing it in short OHCs. In a companion study, Skellett et al. (1996) demonstrated that this same noise exposure resulted in a significant reduction in DPOAEs. These results suggest that ATP receptor proteins in the cochlea may behave similarly to other receptors by being up- or down-regulated or modulated during various physiological and environmental conditions. The rat is less sensitive than guinea pig to the effects of intense sound (Borg et al., 1995). Thus, the ionotropic ATP receptor on OHCs of the rat may be modulated or down-regulated to a great extent. This mechanism may desensitize the rat to damaging effects of intense sounds in its environment.

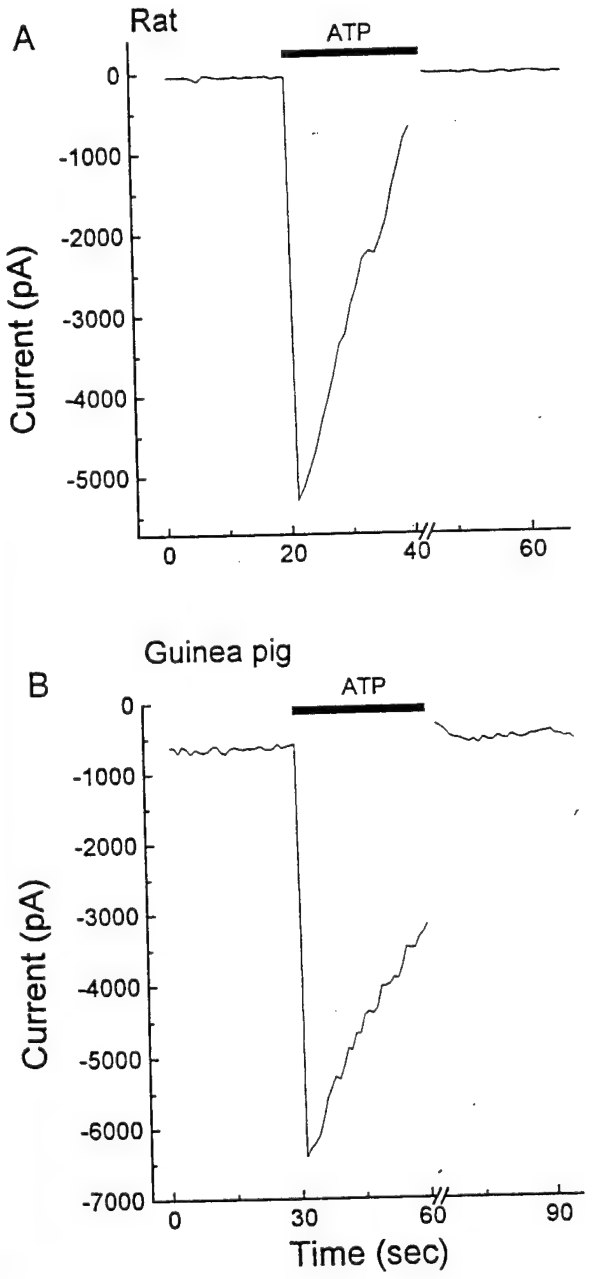

Fig. 5. Examples of an ATP-induced inward current response obtained from a rat Deiters' cell (A) and a guinea pig Deiters' cell (B) at −70 mV. Current–voltage (I–V) relationships were recorded in the absence and presence of 10 μM ATP. The voltage command was ramped from −150 mV to +60 mV (0.5 mV/ms) and the ramp was presented at the rate of one every second. Membrane potential was held at −60 mV. Traces were constructed from the current evoked by the ramp utilizing the current value obtained at −70 mV.

Acknowledgments

Thanks to Latasha Bright and Maureen Fallon for technical assistance and Charles H. Norris, Anastas Nenov and Ruth A. Skellett for advice. This work was supported in part by research grant number 5 R01 DC 00722-05 from the National Institute on Deafness and Other Communication Disorders, National Institutes of Health, and DAMD 17-93-V-3013, Kam's fund for Hearing Research, and the Louisiana Lions Eye Foundation.

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Appendix #8
DAMD17-93-V-3013
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Hearing Research 111 (1997) 42–54

HEARING RESEARCH

Pharmacological evidence that endogenous ATP modulates cochlear mechanics

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Received 14 March 1997; revised 22 May 1997; accepted 22 May 1997

Abstract

In the cochlea, outer hair cells (OHCs) and Deiters' cells most likely contribute to the generation of active cochlear mechanics. The presence of ATP receptors on these cells indicates that endogenous ATP may have a role in cochlear mechanics. To explore this possibility, the effects of ATP antagonists were studied both in vivo on distortion product otoacoustic emissions (DPOAEs) using cochlear perfusion and in vitro on isolated OHCs and Deiters' cells using the whole-cell configuration of the patch-clamp technique. Results show that extracellular application of 5–10 μM ATP to OHCs and Deiters' cells induced an inward current that was reduced by both suramin (100 μM) and cibacron (100 μM). Cibacron reduced the voltage gated currents in Deiters' cells and increased them in OHCs, while suramin had no effect. In addition, cibacron induced a hyperpolarizing shift of the half activation voltage of the whole cell currents in Deiters' cells. Suramin (0.1–1 mM) reversibly suppressed the 'slow decline' in the quadratic DPOAE that occurs during continuous stimulation with moderate level primaries. This effect of suramin may be evidence that endogenous ATP alters active cochlear mechanics.

Keywords: Ion channel; Voltage clamp; Otoacoustic emission; Cibacron blue; Suramin; Deiters' cells; Outer hair cells

1. Introduction

Adenosine triphosphate (ATP) has been suggested to subserve both neurotransmitter and neuromodulator functions in a number of neuronal systems including the cochlea (see reviews: Burnstock, 1990; Bobbin, 1996; Eybalin, 1993). Bobbin and Thompson (1978) first suggested ATP as a candidate for a neurotransmitter or neuromodulator in the cochlea based on its relative potency in reducing the compound action potential of the auditory nerve. Kujawa et al. (1994a) demonstrated that ATP-γ-S, an analogue of ATP that is not rapidly metabolized, was one of the most potent compounds studied in affecting cochlear potentials when instilled into the perilymph compartment of guinea pig cochleas. ATP and some of the other analogues tested (e.g. 2-methylthio-ATP) were less effective probably because they were rapidly metabolized (Kujawa et al., 1994a; Kennedy and Leff, 1995; Vlajkovic et al., 1996). Among the many effects of ATP-γ-S in the cochlea was that it abolished cubic distortion product otoacoustic emissions (DPOAEs). Active mechanical transduction processes in the cochlea generate DPOAEs (Bobbin, 1996; Brownell, 1996; Frank and Kossl, 1996). Therefore, the suppression of the DPOAEs by ATP-γ-S suggests that activation of ATP receptors in the cochlea has powerful effects on active cochlear mechanics.

ATP-γ-S most likely acts on the ATP receptors in the organ of Corti and not on those in the stria (Suzuki et al., 1995; Wangemann, 1995; White et al., 1995) because the drug had little effect on the endocochlear potential (Kujawa et al., 1994a,b). In the organ of Corti, OHCs, inner hair cells and supporting cells have ionotropic ATP receptors (Ashmore and Ohmori, 1990; Chen et al., 1995a,b, 1997; Dulon et al., 1993; Dulon, 1995; Housley et al., 1992; Kakehata et al.,

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PII S0378-5955(97)00093-2

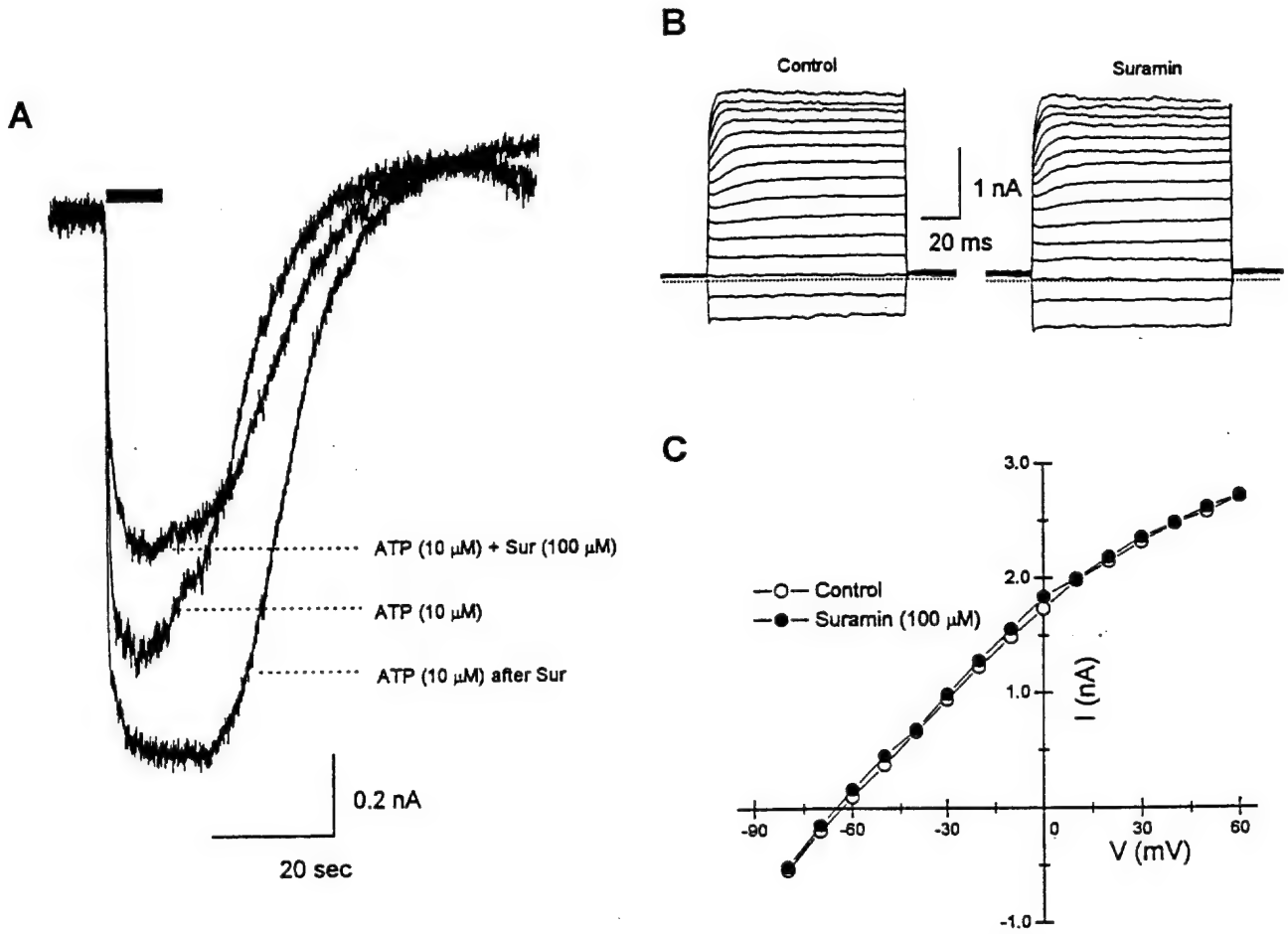

Fig. 1. Effects of suramin (Sur) on ATP gated and voltage activated currents in OHCs. A: ATP (10 μM) induced inward current was suppressed by co-application with suramin (100 μM) and was enhanced when applied immediately after washing out the co-applied drugs (Vh, −60 mV). B: Current traces recorded from an OHC in the absence and presence of suramin (100 μM; Vh, −60 mV). Dotted lines represent zero current level. C: Current-voltage (I-V) relationships from the same cell as in B. The current was measured 10 ms after the onset of the pulses.

1993; Kujawa et al., 1994b; Nakagawa et al., 1990; Nilles et al., 1994; Sugasawa et al., 1996). The active movement of the outer hair cells (OHCs) which are coupled to Deiters' cells most likely produces DPOAEs (Bobbin, 1996; Brownell, 1996; Frank and Kossl, 1996). Therefore it is possible that the actions of exogenous ATP and related analogues on DPOAEs are due to activation of these receptors on OHCs and Deiters' cells. In addition, tight junctions between the cells forming the borders of scala media separate perilymph from endolymph (Salt and Konishi, 1986) and highly charged compounds such as ATP are unlikely to penetrate through these junctions. Thus, if the ATP receptors on OHCs are on the endolymph side of the OHCs as suggested by others (Housley et al., 1992; Mockett et al., 1994, 1995; Munoz et al., 1995a,b), the effects of ATP and ATP agonists placed in perilymph must be due to their actions on cells other than OHCs, such as Deiters' cells.

The results with agonists applied to the cochlea present evidence as to the effects of activation of ATP receptors but do not demonstrate that endogenous ATP has a similar role in normal physiological function. To demonstrate the role of endogenous neuromodulators or neurotransmitters, pharmacologists have traditionally relied on testing whether antagonists block the physiological response. If the antagonist blocks the response, then this satisfies one criterion in proving that the physiological response is due to that substance (e.g. Bobbin et al., 1985). Kujawa et al. (1994b) demonstrated that the ATP antagonist cibacron blue (cibacron; Collo et al., 1996; Burnstock and Warland, 1987) abolished the cubic DPOAE. In contrast suramin, another ATP antagonist, had no effect on this DPOAE. The results with cibacron may be interpreted as preliminary evidence that cibacron blocked the actions of endogenous ATP at receptors on cells in the organ of Corti, such as Deiters' cells, which prevented the production of normal DPOAEs.

On the other hand, the lack of effect of suramin on the cubic DPOAE may be taken as evidence that cibacron had effects other than blockade of ATP receptors

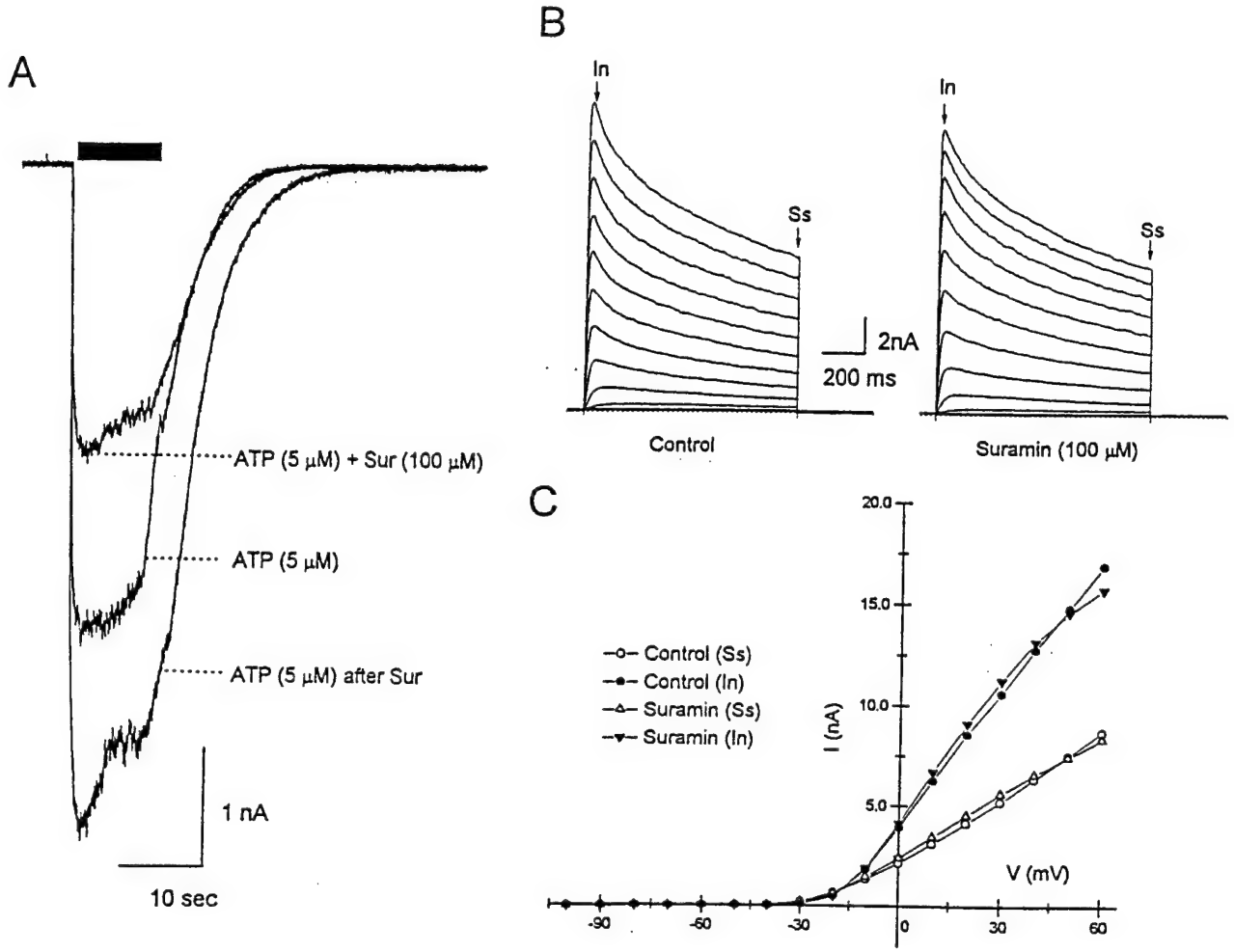

Fig. 2. Effects of suramin (Sur) on ATP gated and voltage activated currents in Deiters' cells. A: ATP (5 μM) induced inward current was suppressed by co-application with suramin (100 μM) and was enhanced when applied immediately after washing out the co-applied drugs (Vh, −80 mV). B: Current traces recorded from a Deiters' cell in the absence and presence of suramin (100 μM; Vh, −80 mV). C: Current-voltage (I-V) relationships were constructed from the steady state (Ss) and instantaneous (In) values measured at the arrows indicated in B.

(Kujawa et al., 1994b). To date there are only a few studies that have examined the pharmacology of ATP agonists or antagonists at the level of the single cells in the organ of Corti (Nakagawa et al., 1990; Sugasawa et al., 1996). Therefore, one purpose of the present study was to test whether there are pharmacological effects of cibacron, other than blockade of ATP, that could account for the powerful effects of cibacron on the cubic DPOAE.

In addition, we pursued further our hypothesis that ATP may have a neuromodulatory role in cochlear mechanics by testing the effects of suramin on the quadratic DPOAE. The quadratic DPOAE is possibly a more sensitive indicator of active cochlear mechanics than the cubic DPOAE (Frank and Kossl, 1996). The quadratic DPOAE undergoes complex time varying changes in amplitude when monitored over minutes during continuous sound stimulation with moderate level primaries (Brown, 1988; Kirk and Johnstone, 1993; Kujawa et al., 1995, 1996; Lowe and Robertson, 1995). The efferent neurotransmitters, acetylcholine and gamma-aminobutyric acid, have been shown not to have a role in this amplitude change (Kujawa et al., 1996; Kirk and Johnstone, 1993; Lowe and Robertson, 1995). Therefore, we examined the effect of suramin perfusion of the perilymph compartment on the time varying amplitude changes of the quadratic DPOAE. Preliminary results have been presented (Bobbin et al., 1997).

2. Methods

2.1. In vitro procedures

2.1.1. Isolation of OHCs and Deiters' cells

OHCs and Deiters' cells from pigmented guinea pigs were isolated as described previously (Chen et al., 1995a,b). Animals were anesthetized with pentobarbital (35 mg/kg, i.p.), decapitated, and the bulla separated

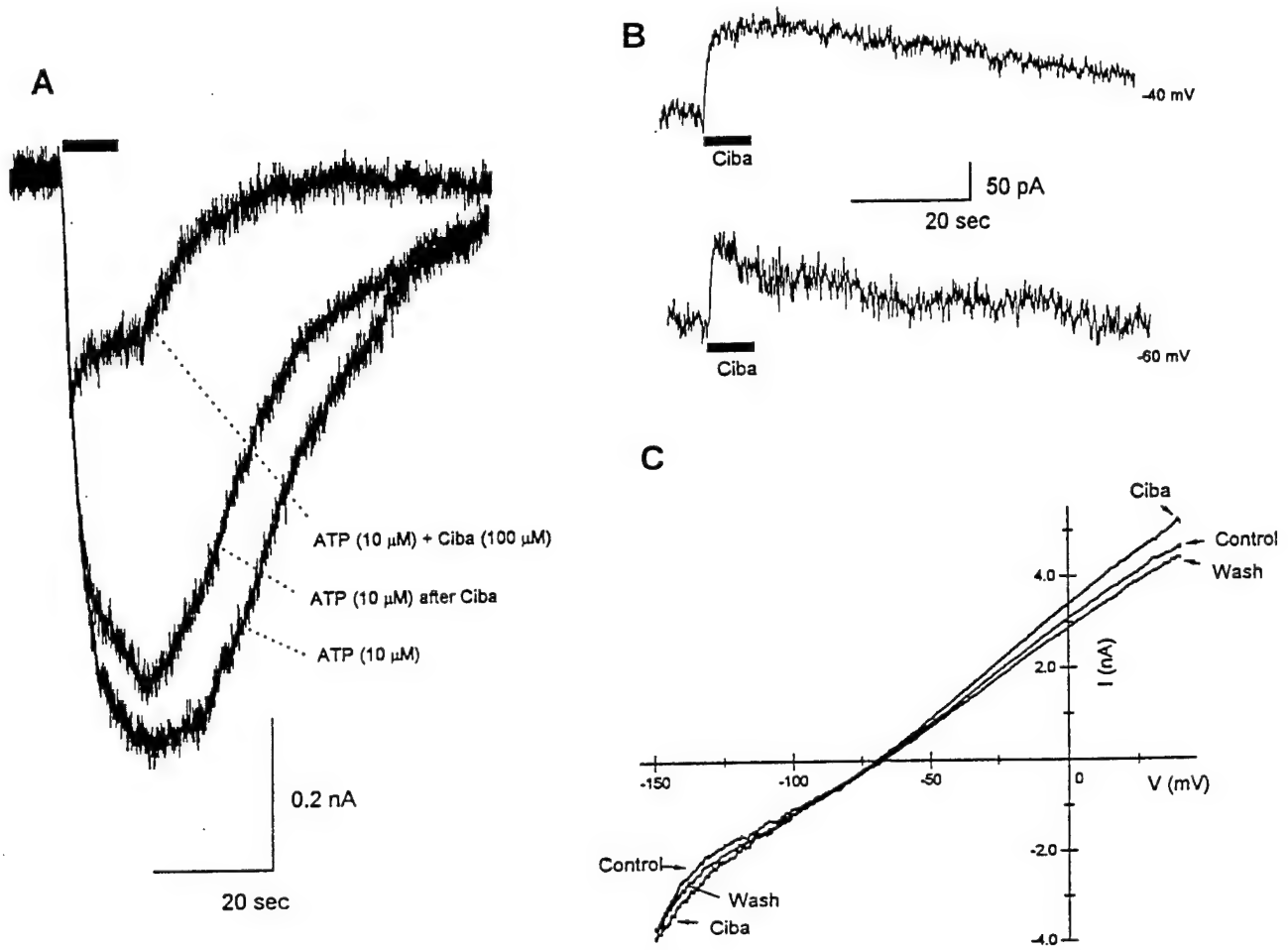

Fig. 3. Cibacron (Ciba) suppressed the ATP gated current and increased whole cell current in OHCs. A: ATP (10 μM) induced inward current was suppressed by co-application with cibacron (100 μM; Vh, −60 mV). B: Cibacron (100 μM) induced an outward current at Vh of −60 mV and −40 mV. C: Current-voltage (I-V) curves recorded from an OHC before (control), during (Ciba) and after washing out (wash) 100 μM cibacron. The voltage command was ramped from −150 mV to +40 mV (0.38 mV/ms; Vh, −60 mV).

and placed in a modified Hanks' balanced saline (HBS). The bone surrounding the cochlea was removed, and the organ of Corti was placed in 200 μl of HBS containing collagenase (1 mg/ml, Type IV, Sigma) for 5 min. The cells were then isolated and transferred into the dishes containing a 150 μl drop of HBS using a microsyringe and stored at room temperature. The length of each cell was measured with a calibrated reticulum prior to recording.

2.1.2. Whole-cell voltage clamp

Single dispersed guinea pig OHCs and Deiters' cells were voltage or current clamped using the whole-cell variant of the patch-clamp technique (Hamill et al., 1981) with Axopatch 200A patch-clamp amplifiers (Axon Instruments). Patch electrodes were fabricated from borosilicated capillary tubing (Longreach Scientific Resources) using a micropipette puller (Sutter Instrument Co.), and fire polished on a microforge (Narashige Scientific Instrument Lab.) prior to use. Membrane currents were filtered at 5 kHz (−3 dB) using a four-pole low-pass Bessel filter digitized with a 12-bit A/D converter (DMA Interface, Axon Instruments), and stored for off-line analysis using a pentium microcomputer. Voltage paradigms were generated from a 12-bit D/A converter (DMA Interface, Axon Instruments) using pClamp software (Axon Instruments). After establishment of the whole-cell configuration, series resistance and cell capacitance compensation were carried out prior to recording with 80% series resistance compensation normally applied. No substraction of leakage current was made.

2.1.3. Solutions

The HBS utilized for isolating cells and perfusing the bath contained (in mM): NaCl, 145; KCl, 5.4; $CaCl_2$, 2.5; $MgCl_2$, 0.5; HEPES, 10; and glucose, 10. The HBS solution was adjusted to a pH of 7.40 with NaOH and to 300 mOsm/kg H_2O with sucrose. The K^+ internal solution contained (in mM): KCl, 140; $MgCl_2$, 0.5; HEPES, 5; EGTA, 11; $CaCl_2$, 0.1; Na_2ATP, 2; and Na_2GTP, 0.1. The internal solution was adjusted to a

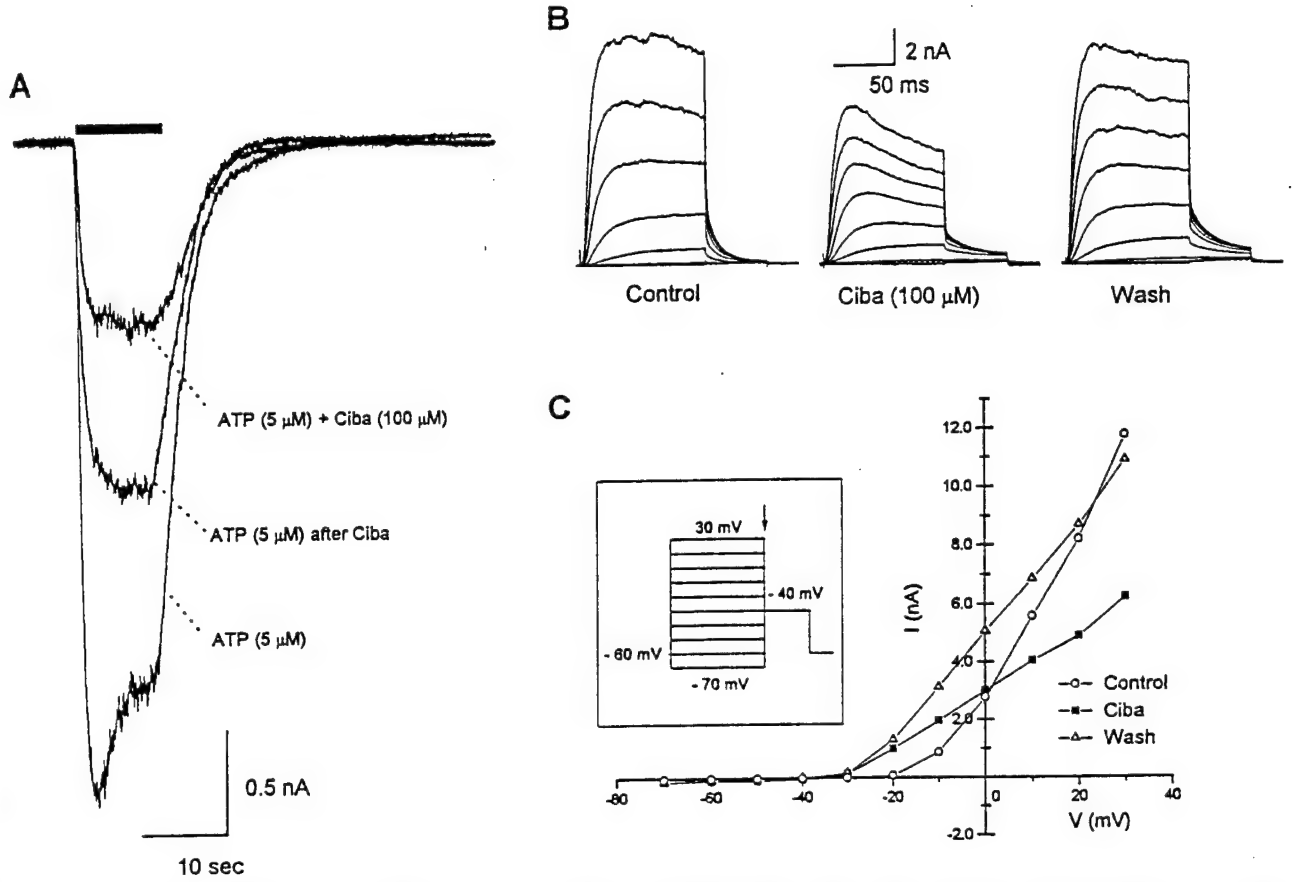

Fig. 4. Cibacron (Ciba) blocked the ATP gated current and inhibited voltage dependent whole cell current in Deiters' cells. A: ATP (10 μM) induced inward current was suppressed by co-application with cibacron (100 μM; Vh, −80 mV). B: Current traces recorded from a Deiters' cell before (control), during and after washing out (wash) cibacron (100 μM). The voltage protocol is as shown in inset. C: Current-voltage (I-V) curves constructed from the current records of the same cell as in B. Current amplitude was measured 1 ms before the termination of the pulses as indicated by an arrow.

pH of 7.35 with HCl and had an osmolality of 284 mOsm/kg H_2O adjusted with sucrose. Low (free) Ca^{2+} internal solution contained (in mM): K^+, 137; Mg^{2+}, 1.5; Ca^{2+}, 0.1; Cl^-, 95.2; *N*-methyl-D-glucamine, 60; Na^+, 4; ATP,2; GTP, 0.1; BAPTA, 25 and had an osmolality of 284 mOsm/kg H_2O adjusted with sucrose. The drugs tested were freshly prepared at desired concentrations in the HBS external solution. All the drug solutions were delivered either from a U-tubing system as described previously (Chen et al., 1995a,b) or bath perfusion. All experiments were conducted at room temperature (22–24°C).

2.2. *In vivo procedures*

2.2.1. *Subjects*

Experiments were performed on pigmented guinea pigs of either sex weighing 250–400 g. Anesthetized animals (urethane, Sigma; 1.5 g/kg, i.p.) were tracheotomized and were allowed to breathe unassisted. ECG and rectal temperature were monitored throughout each experiment and temperature was maintained at 38 ± 1°C by a heating pad. Additional urethane was administered as required to maintain an adequate depth of anesthesia. Surgical procedures have been described previously (Kujawa et al., 1994a,b). In all animals the right auditory bulla was exposed using a ventrolateral approach and tendons of the right middle ear muscles were sectioned.

2.2.2. *Cochlear perfusion experiments*

Perfusions were carried out using methods described previously (Kujawa et al., 1994a,b). The artificial perilymph (AP) had a composition of (in mM): NaCl, 137; KCl, 5; $CaCl_2$, 2; NaH_2PO_4, 1; $MgCl_2$, 1; glucose, 11; $NaHCO_3$, 12. The suramin was mixed with the AP on the day of use at desired concentrations. The pH of all solutions was adjusted to 7.4 when necessary. Perfusates were introduced into the cochlear perilymph at room temperature and at a rate of 2.5 μl/min for 15 min through a hole in basal turn scala tympani and were allowed to flow from the cochlea through an effluent hole placed in basal turn scala vestibuli. Effluent was absorbed within the bulla using small cotton wicks. In all animals, the first two perfusions were of AP alone. These perfusions were performed to achieve a

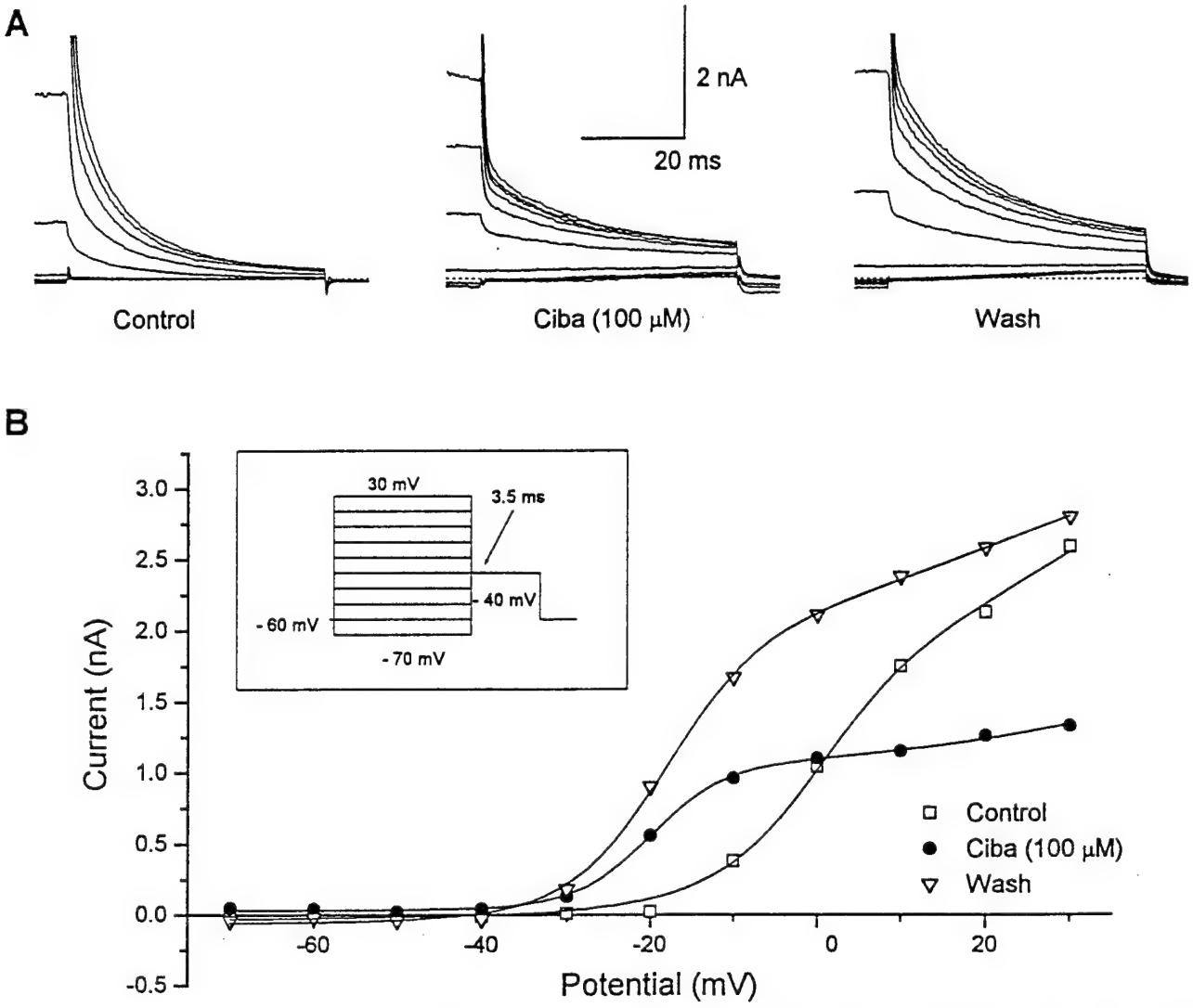

Fig. 5. Tail activation curves of cibacron induced inhibition of voltage gated current in Deiters cells. A: Superimposed tail current traces recorded from a Deiters' cell before (control), during (Ciba) and after (wash) application of cibacron (100 μM). Current elicitation was the same as in Fig. 4. B: Tail activation curves constructed from current records in A. Tail current amplitude was measured 3.5 ms after the termination of the pulses as indicated in inset. The tail activation curves were best fitted to a double Boltzmann function. The parameters for the fits: $V_{h1} = -3.7 \pm 3.2$ mV, $K_1 = 5.4 \pm 0.3$ mV, $V_{h2} = 35.4 \pm 2.7$ mV, and $K_2 = 17.9 \pm 1.2$ mV for the control ($n = 3$); $V_{h1} = -20.7 \pm 1.2$ mV, $K_1 = 5.1 \pm 0.3$ mV, $V_{h2} = 34.3 \pm 2.2$ mV, and $K_2 = 20.8 \pm 2.8$ mV for the cibacron ($n = 3$); $V_{h1} = -15.2 \pm 1.8$ mV, $K_1 = 6.2 \pm 0.2$ mV, $V_{h2} = 27.0 \pm 4.0$ mV, and $K_2 = 17.4 \pm 1.1$ mV for the wash ($n = 3$).

stable baseline to which subsequent alterations in the artificial perilymph and drug-related changes could be compared. These perfusions were followed by perfusions of the AP or experimental drug.

2.2.3. *DPOAE: stimulus generation and response monitoring*

The instrumentation employed in these experiments has been described (Skellett et al., 1996). Briefly, quadratic ($f_2 - f_1 = 1.25$ kHz) and cubic ($2f_1 - f_2 = 5$ kHz) DPOAEs were elicited by equilevel primary stimuli ($f_1 = 6.25$ kHz; $f_2 = 7.5$ kHz) sent to two separate speakers housed within an acoustic probe assembly. The acoustic probe assembly was tightly coupled directly to the right ear of each animal. The DPOAEs were detected by a microphone also housed within the probe assembly and amplified using a microphone preamplifier and sent to a dynamic signal analyzer for fast Fourier transform analysis (averaging 10 discrete spectra) and spectral display (span = 1 kHz; CF = DP frequency; BW = 3.75 Hz). The noise floors associated with these display windows averaged approximately −15 dB SPL for the $f_2 - f_1$ DPOAE and −18 dB SPL for the $2f_1 - f_2$ DPOAE when measured at points ±50 Hz from the DPOAE frequency.

As described previously (Kujawa et al., 1995, 1996), continuous, moderate-level, primary stimulation ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, L1 = L2 = 60 dB SPL) generated a standard pattern of time-varying alterations in the amplitude of the quadratic ($f_2 - f_1 = 1.25$ kHz)

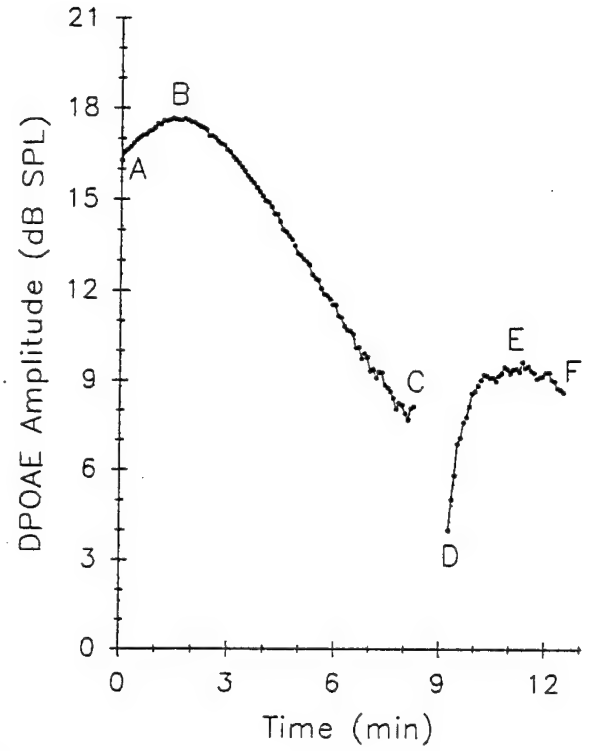

Fig. 6. A typical example of the effect of equilevel (L1 = L2 = 60 dB SPL) primary stimulation on quadratic (f_2-f_1 = 1.25 kHz) DPOAE amplitude (f_1 = 6.25 kHz, f_2 = 7.5 kHz). Each data point represents a 10-spectrum average and required 5 s to complete. The break in response amplitude trace (C–D) represents 1 min during which the primaries were turned off. Points A–F were used to calculate magnitudes of component amplitude changes for statistical analyses. This particular trace was obtained following a second control artificial perilymph perfusion (AP2) and is also shown in Fig 7. For reading ease, symbols occur only at every third data point; connecting lines follow all data points.

DPOAE. The following stimulation and response monitoring protocol was employed to generate this pattern of change. One hundred consecutive 10-spectrum averages of distortion product amplitude were obtained during continuous primary stimulation. Each of these averages required approximately 5 s to complete for a total of 500 s (8.3 min) of stimulation. The primary tones were then simultaneously turned off and there was a 1 min rest from primary stimulation. Following this rest, the primaries were re-introduced and 40 consecutive 10-spectrum averages of distortion product amplitude were obtained (total time approximately 200 s or 3.3 min of stimulation).

At least a 15 min period of rest from primary stimulation separated each test condition from the next. This period was usually the time (15 min) during which the cochlea was perfused. Immediately (within 2 min), after the perfusion was terminated, the primaries were turned on (60 dB SPL) and recording of the time-varying amplitude of the quadratic DPOAE commenced. Following this recording, without any intervening rest, the level of the primaries was increased from 60 to 70 dB SPL and the amplitude growth functions for the quadratic DPOAE obtained by decreasing the intensity of the primaries in 5 dB steps until the noise floor was reached. This was immediately followed by the same procedure (descending primary intensity) to obtain the amplitude growth functions of the cubic DPOAE.

Effects of treatments were quantified using repeated measures analysis of variance (ANOVA) and Student-Newman-Keuls multiple range test or *t*-tests. Drugs used were: suramin hexasodium salt (RBI), cibacron blue (cibacron; Sigma), and adenosine 5′-triphosphate (ATP, Sigma). The care and use of the animals reported on in this study were approved by LSUMC's Institutional Animal Care and Use Committee.

3. Results

3.1. Effects of ATP on OHCs and Deiters' cells

The effects of ATP were tested on OHCs shorter than 65 μm and Deiters' cells. As described previously (Chen et al., 1995a,b, 1997) ATP from 5 to 100 μM evoked inward currents at the holding potential (Vh) in both OHCs (Vh = −60 mV) and Deiters' cells (Vh = −80 mV).

The purinergic receptor antagonist suramin (100 μM) reduced the ATP (10 μM) induced current in OHCs by 19 ± 7% (n = 6) and the ATP (5 μM) induced current in Deiters' cells by 25 ± 6% (n = 10, Fig. 1AFig. 2A). When ATP was applied following the co-application of suramin and ATP (10–20 s later), the responses were larger than those induced by the first ATP application (5 out of 6 OHCs; 8 out of 10 Deiters' cells; Figs. 1A and 2A). This potentiation reversed with washing.

The effect of cibacron on ATP gated current was tested in both OHCs and Deiters' cells. As indicated in Figs. 3A and 4A, 100 μM cibacron decreased the ATP (10 μM) induced current by 72 ± 6% in OHCs (n = 6) and reduced the ATP (5 μM) induced current by 78 ± 2% in Deiters' cells (n = 7). When ATP was applied following the co-application of cibacron and ATP (10–20 s later), the responses were still smaller than those induced by the first ATP application (OHCs, n = 4; Deiters' cells, n = 7; Figs. 3A and 4A).

The effects of suramin and cibacron on whole cell currents were monitored in OHCs and Deiters' cells. Suramin had no effect on the voltage gated currents in OHCs (Fig. 1B,C, n = 14) or Deiters' cells (n = 9, Fig. 2B,C). However, cibacron reversibly increased these currents in OHCs (Fig. 3), but decreased them in Deiters' cells (Fig. 4). As indicated in Fig. 3B, cibacron induced an outward current at Vh of −60 and −40 mV, and an inward current at Vh of −100 mV (data not shown) in OHCs (n = 5). The reversal potential of the cibacron induced current is close to the po-

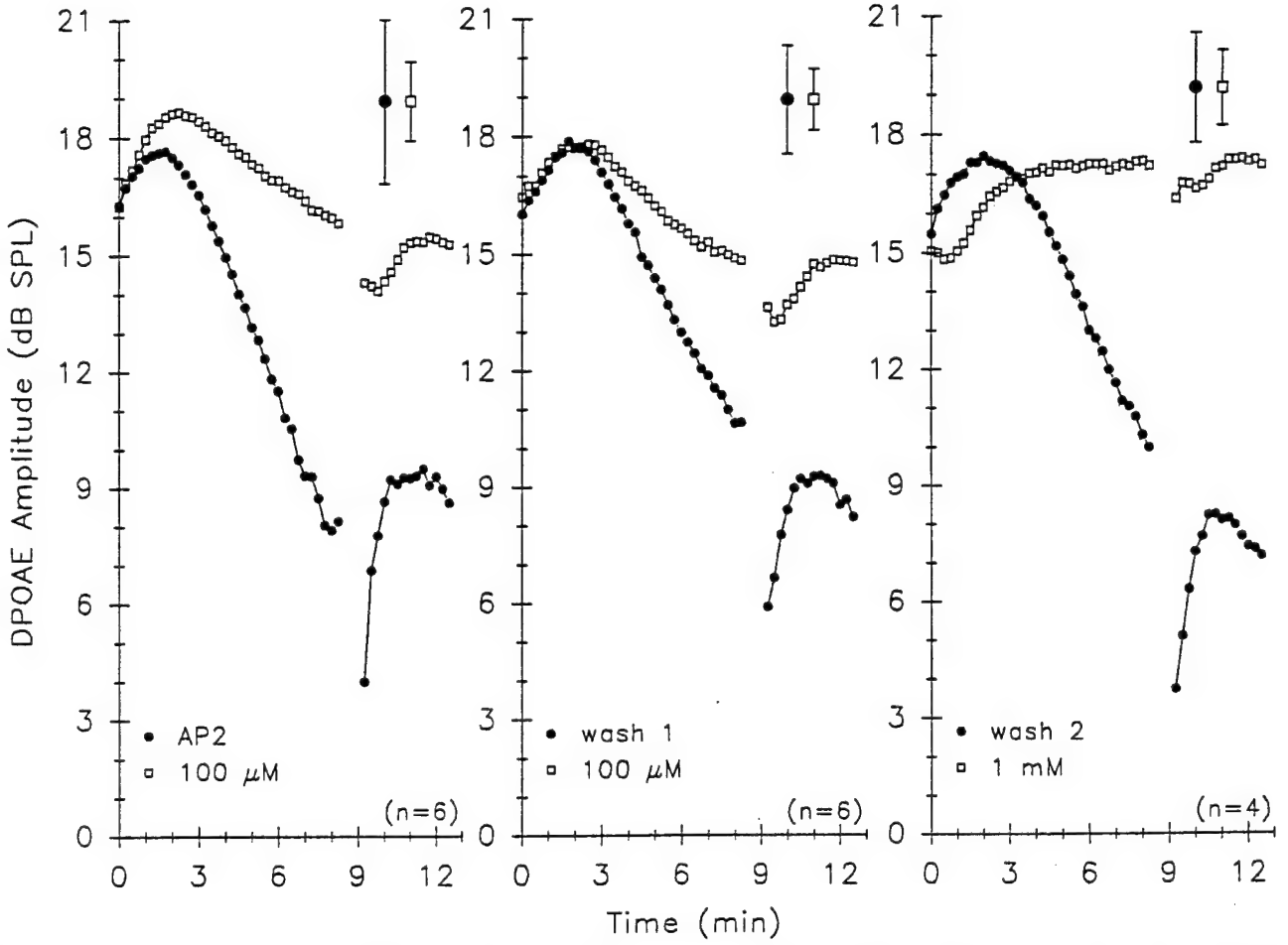

Fig. 7. Effect of suramin on f_2-f_1 DPOAE amplitude alterations during continuous primary stimulation ($f_1=6.25$ kHz, $f_2=7.5$ kHz, L1 = L2 = 60 dB SPL). Response amplitude as recorded following the second control perfusion (AP2), suramin (100 μM), a following wash (wash 1), a second suramin (100 μM), a second wash (wash 2), and a third suramin (1 mM). Pooled errors are shown in the upper right hand corner of each frame. See legend for Fig. 6 for additional information.

tassium equilibrium potential. The current-voltage (I-V) relationship recorded from a ramp protocol shows that cibacron increased an outward current at potentials positive to −80 mV and an inward current at more negative potentials (Fig. 3C; 9 out of 11 cells).

In Deiters' cells cibacron induced an inhibition of whole cell current (Fig. 4B,C, $n=12$), and shifted the activation curve of whole cell currents in a hyperpolarizing direction (Fig. 5, $n=3$). The activation curves of voltage activated currents in Deiters' cells are likely to represent the activity of a single channel type (data not shown) and were best fit to curves described by double Boltzmann equation. The estimated parameters for the half activation voltages and the dependence of the activation on voltage were obtained from the fitted curves and are shown in Fig. 5 (legend). The larger current component activated at more hyperpolarized potentials and was more steeply dependent on voltage than the smaller component. Overall, cibacron caused a concentration dependent hyperpolarizing shift of the larger current component (−3.7 ± 3.1 mV to −20.7 ± 1.2 mV, mean ± S.E.; $n=3$; $P<0.05$ independent t-test) but not of the smaller component (35.4 ± 2.7 mV to 34.3 ± 2.2 mV, mean ± S.E.; $n=3$; $P>0.05$ independent t-test). The slope of the activation currents remained unchanged (Fig. 5). The hyperpolarizing shift of the half activating voltage of the larger current component could not be reversed by HBS wash of more than 30 min.

To investigate the possibility that the hyperpolarizing shift of half activation voltage is a time related phenomenon rather than drug induced, we applied HBS to cells for 15 min continuously and recorded the currents ($n=10$). Under these conditions, in the absence of cibacron, hyperpolarization of the activation of the currents was not observed. In addition, hyperpolarization always coincided with the application of the drug and was independent on the time of the application (between the sixth and fifteenth min after establishing a whole cell mode of recording). These effects of cibacron were concentration dependent as evidenced by the similar changes induced by application of 65 μM cibacron

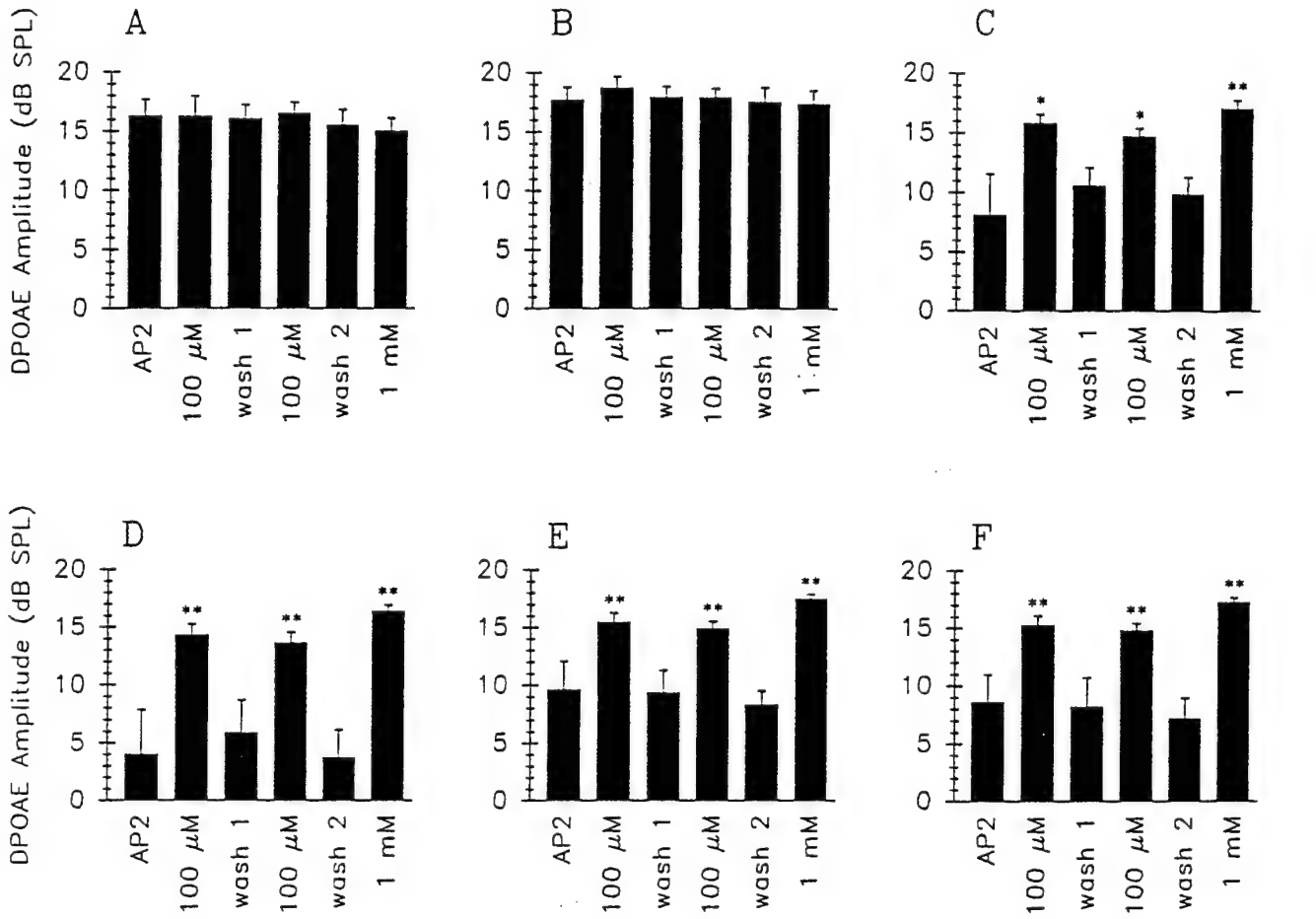

Fig. 8. Effect of suramin on the various points (A–F) identified in the f_2-f_1 DPOAE amplitude values (defined in Fig. 6) following each perfusion shown in Fig. 7. Significance was tested with ANOVA and subsequent Student-Newman-Keuls post hoc multiple range test for all means except the wash 2 vs. 1 mM suramin means which were compared only to each other by a *t*-test. Values that are significantly different from their respective AP2 concentrations (or in the case of 1 mM suramin, its respective wash 2) are designated: $^*P<0.05$; $^{**}P<0.01$. Data are represented as means ± S.E. (for *n* see Fig. 7).

blue to the Deiters' cells ($n=2$; shift of the activation of the larger current component of 6.5 and 8.3 mV for each cell tested, respectively). Similar changes were observed when Deiters' cell currents were recorded using an internal solution containing 25 mM BAPTA ($n=2$).

In summary, cibacron blue changes the voltage induced whole cell currents of OHCs and Deiters' cells while suramin had no effect. These effects were in addition to the block of the purinergic response.

3.2. *Effects of suramin on DPOAEs*

A typical example of the effect of continuous low level primary stimulation on quadratic (f_2-f_1) DPOAE amplitude is shown in Fig. 6. Six points (A–F) were identified on each response amplitude function (Fig. 6). These values were used to characterize the response as previously described (Kujawa et al., 1996). They include an initial value (A), an 'on effect' (A–B), a 'slow decline' (B–C), an 'off effect' (C–D) and a 'second on effect' (D–E). In preliminary experiments, suramin was perfused through the cochlea in a cumulative dose manner and was found to reversibly attenuate the 'slow decline' in the quadratic DPOAE induced by continuous, moderate primary stimulation ($n=4$ animals). The effect of suramin was detected at a threshold concentration of around 10–33 μM and the 'slow decline' in the quadratic DPOAE amplitude was almost totally abolished after exposure to 1 mM suramin.

To quantitate this effect of suramin, examine the test-retest and reversibility of the drug, and to describe the effect on the amplitude growth function of the DPOAEs, in 4–6 additional animals, we perfused the cochlea in the following order: AP twice, 100 μM suramin, an AP wash, 100 μM suramin, an AP wash, and 1 mM suramin. Fig. 7 illustrates the effects of repeated 100 μM suramin perfusions on the quadratic DPOAE amplitude changes, the complete reversibility of the drug upon washing, and the total abolishment of the 'slow decline' in the quadratic DPOAE amplitude by the 1 mM suramin.

Suramin had no significant ($P>0.05$) effect on the

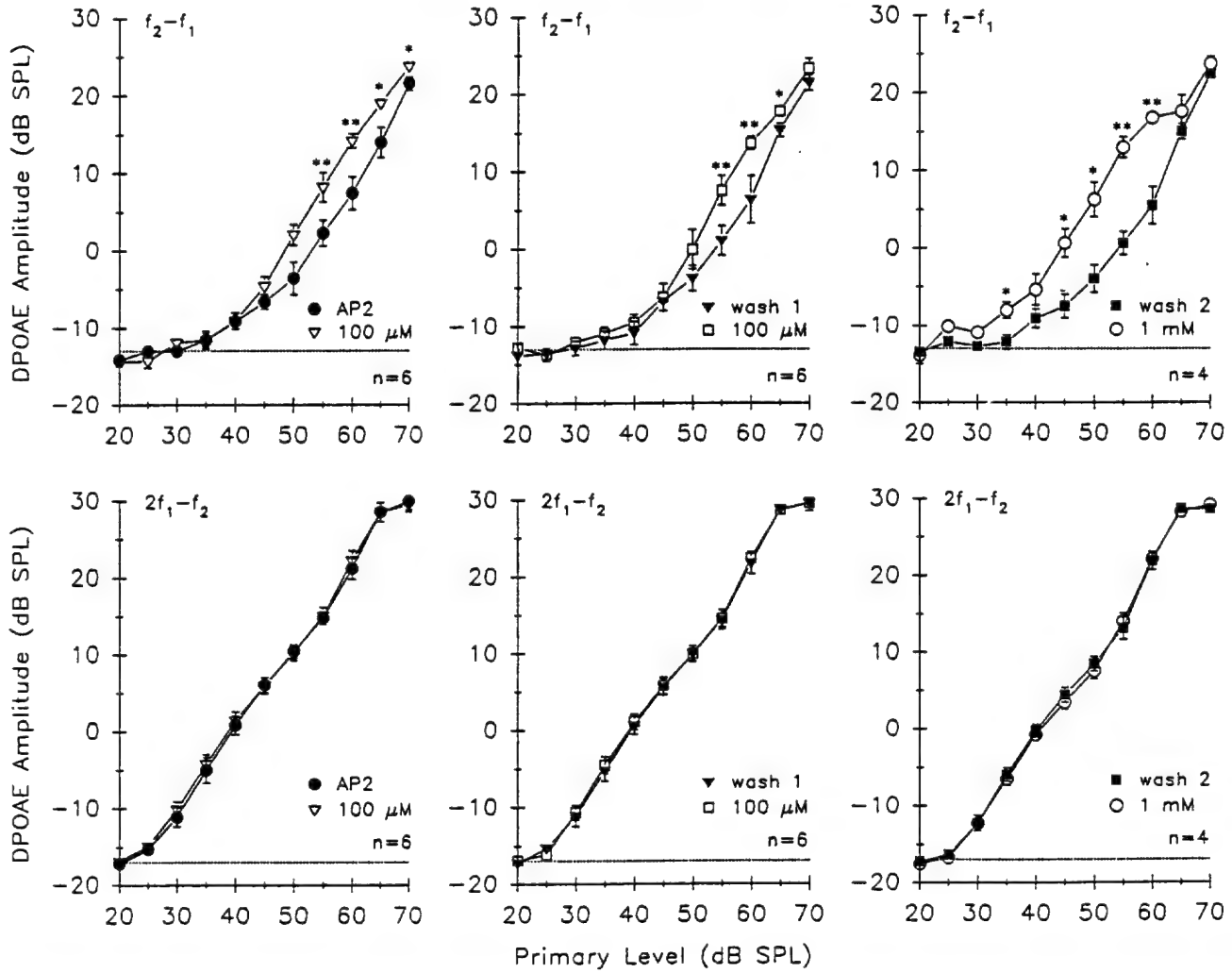

Fig. 9. Effect of suramin on the amplitude growth functions for quadratic (f_2-f_1) and cubic ($2f_1-f_2$) DPOAEs. Shown are the values obtained following the second control perfusion (AP2), suramin (100 μM), a wash out (wash 1), a second suramin (100 μM), a wash out (wash 2) and a third suramin (1 mM) and collection of the data shown in Fig. 7. Data are represented as means ± S.E. Significance was tested with ANOVA and subsequent Student-Newman-Keuls post hoc multiple range test for all means except wash 2 vs. 1 mM suramin means which were compared only to each other by a *t*-test. Values that are significantly different from their respective AP2 concentrations (or in the case of 1 mM suramin, its respective wash 2) are designated: $^*P<0.05$; $^{**}P<0.01$. The dashed line in each panel represents the average value of the noise floor.

absolute amplitude of the quadratic distortion product measured after the 15 min of silence at point A (Fig. 8). Also, suramin did not substantially alter the 'on effect' (B; Fig. 8). On the other hand, it did decrease the slope of the 'slow decline' as indicated by the increase in point C (Fig. 8). The decrease in the 'slow decline' probably also accounted for the increase in points D–F (Fig. 8).

The amplitude growth functions for the quadratic DPOAE obtained immediately after the above measurements following the 100 μM and the 1 mM suramin perfusions were characterized by a significant shift in the function to the left compared to those obtained after the washes (Fig. 9). This reflects the suramin induced attenuation of the 'slow decline' in the quadratic DPOAE during continuous exposure to the primaries. In contrast, there was no change in the amplitude growth functions of the cubic DPOAE (Fig. 9), confirming our previously published results (Kujawa et al., 1994b).

4. Discussion

Results demonstrate that both suramin and cibacron reduce ATP gated currents in acutely isolated OHCs and Deiters' cells. The potentiation of ATP induced responses after suramin may have been due to one of the other effects of suramin, such as blockade of ecto-ATPase (Ziganshin et al., 1995) or inhibition of diacyl-

glycerol kinase (Kopp and Pfeiffer, 1990). Suramin had no effect on voltage gated currents in either OHCs or Deiters' cells.

In OHCs, cibacron induced an outward current at the holding potential of −60 mV and increased the whole cell currents. The shape and reversal potential of the currents induced by cibacron were similar to those induced by the rise in intracellular Ca^{2+} in inner hair cells (Dulon et al., 1995). This similarity may suggest that cibacron increases the level of free internal Ca^{2+} which in turn affects Ca^{2+} sensitive K^+ currents such as K_n or K_{Ca}. Cibacron has been shown to activate Ca^{2+} dependent potassium currents in bladder smooth muscle (Cotton et al., 1996) and our results are consistent with such a mechanism of action of cibacron in OHCs.

In Deiters' cells, cibacron reduced the voltage gated currents and induced a hyperpolarizing shift of the half activation voltage of the whole cell currents. Internal calcium appears not to have a role in this effect since it was also observed in the presence of high internal BAPTA. Thus the mechanism for the action of cibacron on Deiters' cells is unknown.

Cibacron's effect on voltage activated currents in OHCs and Deiters' cells, cells which are involved in generating otoacoustic emissions (Bobbin, 1996; Frank and Kossl, 1996; Brownell, 1996), probably caused, or contributed to, the large decrease in the cubic DPOAEs reported by us previously (Kujawa et al., 1994b). The lack of effect of suramin on the same voltage activated currents may explain its lack of effect on both the cubic DPOAE (Kujawa et al., 1994b) and on the initial value of the quadratic DPOAE found in the present study (point A in Figs. 6 and 8).

Several investigators have attempted to discover the mechanisms underlying the time varying amplitude changes of the quadratic DPOAE that occur during continuous stimulation with moderate level primaries. Kirk and Johnstone (1993) suggested that the amplitude changes were due to GABA released by the efferents synapsing onto the OHCs. Lowe and Robertson (1995) demonstrated conclusively that the efferents do not contribute to the amplitude change. Similar conclusions were reached by Kujawa et al. (1995, Kujawa et al., 1996) who demonstrated that the amplitude change was not affected to a large extent by: TTX, a sodium channel antagonist; curare in concentrations that blocked the action of acetylcholine released by the efferents onto the OHCs; bicuculline, a GABA antagonist; and efferent sectioning, but was affected by alterations in calcium. In the present study, suramin reversibly suppressed the 'slow decline' in amplitude of the quadratic DPOAE that occurs in response to continuous primary presentation, indicating that endogenous release of ATP, acting on ATP receptors, may underlie this 'slow decline' in amplitude. Since suramin had no effect on the initial value (point A) or on the 'on effect' (point B) of this time varying amplitude change, then ATP may only be involved in generating the 'slow decline' (B–C; Figs. 6 and 8).

One must be circumspect in attributing the effect of suramin on the quadratic DPOAE to blockade of endogenous ATP as suramin has been reported to have many effects other than blockade of ATP at ATP receptors. For instance, suramin has been reported to inhibit the ectonucleotidases that degrade ATP (Bailey and Hourani, 1994; Ziganshin et al., 1995). Suramin also inhibits diacylglycerol kinase (Kopp and Pfeiffer, 1990) and several protein tyrosine phosphatases (Ghosh and Miller, 1993). In addition, suramin has an antineoplastic effect (Stein, 1993). Further evidence as to the mechanism by which suramin affects the quadratic DPOAE may be obtained by examining the effects of additional ATP antagonists in future studies of this phenomena.

In this study we have focused on cochlear mechanics as monitored by DPOAEs. DPOAEs are generated by the active cochlear mechanics, the cochlear amplifier, brought about by the change in length of the OHCs coupled to Deiters' cells (e.g. Brownell, 1996; Bobbin, 1996; Frank and Kossl, 1996). Both OHCs and Deiters' cells have ATP receptors in their membranes. However, as discussed in the introduction, some investigators suggest that ionotropic ATP receptors on OHCs are located near the stereocilia portion of the cells or on the endolymph side of the cells (Housley et al., 1992; Mockett et al., 1994, 1995). If this is the case, then only ATP released onto Deiters' cells may be affected by suramin which was placed in perilymph and therefore bathed the basal and lateral walls but not the cuticular plate region of the OHCs. Recently, Wangemann (1996) demonstrated release of ATP from cells of the isolated organ of Corti, presumably in the vicinity of Deiters' cells.

The source of the ATP acting on ATP receptors on Deiters' cells (and possibly OHCs) is unknown and the effects of this interaction at the cellular level is speculative at present. Since TTX, which blocks action potential generation, and efferent sectioning have no effect on the quadratic DPOAE amplitude change (Lowe and Robertson, 1995; Kujawa et al., 1995), we assume that the efferent nerve fibers have no role in this phenomena. ATP opens ligand gated cation channels on Deiters' cells resulting in the depolarization of these cells. This depolarization, together with a possible change in Ca^{2+} levels in the Deiters' cells (Dulon, 1995; Dulon et al., 1994), may change the tension or stiffness that Deiters' cells apply to the base and apex of OHCs. This change in tension may adjust the operating point of the active cochlear amplifier as described by Frank and Kossl (1996). The cubic DPOAE is less sensitive to changes in the operating point of the cochlear amplifier (Frank

and Kossl, 1996) and may not be affected by the quantity of endogenously released ATP, thus accounting for the lack of effect of suramin on this DPOAE. However, the cubic DPOAE is suppressed by exogenously applied ATP and ATP agonists (Kujawa et al., 1994a). This probably occurred because the large amount of ATP applied induced a stimulation of the ATP receptors on Deiters' cells that was sufficiently large to alter the cubic DPOAEs.

In summary, suramin and cibacron blocked the cationic inward currents evoked by application of ATP to OHCs and Deiters' cells. Cibacron but not suramin, increased the voltage activated outward currents in OHCs and decreased them in Deiters' cells. Given the fact that suramin did not affect the cubic DPOAE, it appears that the cibacron induced suppression of the cubic DPOAE reported earlier (Kujawa et al., 1994b) may have been due to the actions of cibacron on voltage activated currents in OHCs and Deiters' cells. In vivo, suramin attenuated the 'slow decline' in the amplitude of the quadratic DPOAE induced by moderate, continuous primary stimulation without an effect on the initial value or the 'on effect'. The suramin results obtained in vivo suggest that endogenous ATP may play a role in generating this 'slow decline'. This decrease in amplitude may reflect an ATP induced adjustment of the operating point of the cochlear amplifier.

Acknowledgments

Thanks to Latasha Bright and Julie Campbell for technical assistance. This work was supported in part by Research Grants R01 DC 00722 and PO1 DC00379 from the National Institute on Deafness and Other Communication Disorders, National Institutes of Health, and DAMD 17-93-V-3013, Kam's Fund for Hearing Research, and the Louisiana Lions Eye Foundation.

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Hearing Research 000 (1997) 1–10

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Cytotoxicity and mitogenicity of adenosine triphosphate in the cochlea

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Received 23 April 1997; revised 11 July 1997; accepted 23 July 1997

Abstract

Evidence is accumulating to indicate that extracellular adenosine 5′-triphosphate (ATP) may function as a neurotransmitter, neuromodulator, cytotoxin and mitogen. Many of the cells in the cochlea have ATP receptors, however, their function is unknown. The purpose of the present study was to test whether ATP may act as a cytotoxin in the cochlea. ATP was applied to acutely isolated outer hair cells (OHCs) and their shape changes monitored. In addition, ATP was applied into the cochlea by perfusion of the perilymph compartment for 2 h and the animals allowed to survive 3–4 weeks post drug application. At this time, sound-evoked cochlear potentials and distortion product otoacoustic emissions (DPOAEs) were monitored and the cochleas evaluated histologically. Results indicate that when applied to isolated OHCs, ATP (3–30 mM) induced a bleb formation in the infracuticular region of the cell that burst within a few minutes. Short OHCs were more sensitive to this effect of ATP than long OHCs. 3–4 weeks after the perilymph perfusion of ATP (60 mM; 2 h) cochlear potentials and DPOAEs were abolished, and histologically, cells in the organ of Corti and the stria vascularis were found to have been destroyed. In addition, there was loss of spiral ganglion cells and proliferating connective tissue filled varying proportions of the scala tympani and vestibuli. Application of sodium gluconate, a control, at the same concentrations had no effect either on the isolated OHCs or when applied in vivo. Results suggest that extracellular ATP or a metabolic product may act as a cytotoxin to some epithelial and neural elements in the cochlea and possibly as a mitogen to mesenchymal cells or fibrocytes.

Keywords: Receptor; Cochlear potential; Cytotoxin; Histology; Fibrocytes; Mitogen

1. Introduction

Extracellular adenosine 5′-triphosphate (ATP) has been suggested to subserve a neurotransmitter, neuromodulator and mitogen function in a number of neuronal and non-neuronal systems (Burnstock, 1990; Collo et al., 1996; Huang et al., 1993; Wang et al., 1994). Others suggest that ATP functions as a toxin being released by cytotoxic T lymphocytes to kill virus-infected hepatocytes (Zoeteweij et al., 1996; Redegeld et al., 1991) and tumor target cells (Gordon, 1986; Di Virgilio et al., 1990). In addition, ATP has been shown to be cytotoxic to mast cells (Tatham and Lindau, 1990) and macrophages (Falzoni et al., 1995). The mechanism for the cytotoxic action is unknown but appears to involve necrosis, i.e. activation of ATP receptors on the surface of the cells which allows for the influx of cations and water with a resultant swelling and bursting of the cell (Tatham and Lindau, 1990; Steinberg and Silverstein, 1989).

Bobbin and Thompson (1978) first demonstrated that acute extracellular application of ATP to the cells of the cochlea by perfusion of the perilymph compartment affected the function of the cochlea as monitored by a reduction in the compound action potential of the auditory nerve. Using the same application route, Kujawa et al. (1994a) demonstrated that ATP-γ-S, an analogue of ATP that is not rapidly metabolized, was much more potent than ATP. Studies at the single cell level have demonstrated that ATP activates a ligand gated receptor (ionotropic) on outer hair cells (OHCs) that induces a large inward cation current (Ashmore and Ohmori, 1990; Housley et al., 1992; Kakehata et al., 1993; Kujawa et al., 1994b; Nakagawa et al., 1990) and

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PII S0378-5955(97)00140-8

possibly activates a G protein coupled receptor (metabotropic) to increase intracellular free Ca^{2+} in these cells (Ashmore and Ohmori, 1990; Ikeda et al., 1991; Shigemoto and Ohmori, 1990). Additional evidence for a metabotropic receptor mechanism in the cochlea was obtained (Niedzielski and Schacht, 1992; Ogawa and Schacht, 1993, 1994, 1995). Activation of ATP receptors increases intracellular Ca^{2+} levels in inner hair cells (IHCs; Dulon et al., 1991), Deiters' cells (Dulon et al., 1993) and Hensen's cells (Dulon et al., 1993), depolarizes both Deiters' cells (Dulon, 1995) and IHCs (Sugasawa et al., 1996), and may modulate calcium channels (Chen et al., 1995b). Wangemann (1996) demonstrated Ca^{2+}-induced release of ATP from whole organ of Corti. Other evidence demonstrates that ATP induces changes in the cells of the stria vascularis (Liu et al., 1995; Suzuki et al., 1995; Wangemann, 1995), and the origin of the endogenous ATP that acts on these receptors may be the marginal cells in the stria vascularis (White et al., 1995). ATP occurs in high concentrations in endolymph (Munoz et al., 1995a), and when exogenous ATP is placed in the endolymph, it induces a large decrease in the endocochlear potential (Munoz et al., 1995b).

The function of ATP receptors in the cochlea is unknown (Bobbin, 1996). The receptors on the stria may regulate the endocochlear potential (Suzuki et al., 1995; Wangemann, 1995). The ionotropic receptors on OHCs may be involved in transduction (Housley et al., 1992; Mockett et al., 1994, 1995). The Deiters' cells may respond to an ATP-induced change in Ca^{2+} levels with a change in stiffness and so be involved in cochlea mechanics (Dulon et al., 1993, 1994; Dulon, 1995). The changes observed in distortion product otoacoustic emissions (DPOAEs) induced by ATP agonists (Kujawa et al., 1994a) and antagonists (Bobbin et al., 1997; Kujawa et al., 1994b; Skellett et al., 1997) are further evidence for a role of ATP in cochlear mechanics. On the other hand, Housley et al. (1995) demonstrated that the application of micromolar concentrations of ATP to isolated OHCs, in the whole cell voltage or current clamp mode, activated an ionotropic receptor causing the OHCs to take up such large amounts of cations and water that swelling localized to the infracuticular region of the cell occurred. This suggests that ATP may kill OHCs by a mechanism similar to that in hepatocytes, mast cells and macrophages (Di Virgilio et al., 1990; Falzoni et al., 1995; Gordon, 1986; Redegeld et al., 1991; Steinberg and Silverstein, 1989; Tatham and Lindau, 1990; Zoeteweij et al., 1996).

We tested the hypothesis that ATP may act as a cytotoxin in the cochlea. ATP was applied to acutely isolated OHCs and their shape changes monitored. In addition, ATP was applied into the cochlea by perilymph perfusion for 2 h and the animals allowed to survive 3–4 weeks post drug application. Following this survival period, sound-evoked cochlear potentials and DPOAEs were monitored and the cochleas evaluated histologically. Preliminary results have been presented (Chu et al., 1997).

2. Methods

2.1. In vitro procedures

2.1.1. Cell isolation

Cells were isolated as previously described (Kujawa et al., 1994a; Skellett et al., 1995). Briefly, adult pigmented guinea pigs (300–500 g) were anesthetized with pentobarbital (Nembutal; 35 mg/kg, i.p.) and killed by decapitation. Whole organ of Corti was mechanically isolated and placed in collagenase (type IV) for 5–10 min. A syringe was used to transfer separated OHCs into dishes containing normal external solution. The external solution was a modified standard Hanks' balanced salt (HBS) containing in mM: 137 NaCl, 5.36 KCl, 2.5 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES, 10 D-glucose. The pH was corrected to 7.4 by addition of appropriate amounts of NaOH. The initial osmolality of the solution was 295 mOsm/kg water which was monitored by utilizing a freezing point osmometer (Precision Systems, model 5002). Osmolality was adjusted to 300 ± 2 mOsm/kg water by adding sucrose. Several morphological criteria were used to judge the viability of the cells used in the experiments. These included: cylindrical shape with diameter around 10 μm; no visible movement of cellular organelles; and the appearance of normal turgor.

2.1.2. Single cell morphological procedure

One OHC in each dish was studied as described by Kujawa et al. (1994a) and Skellett et al. (1995). The dishes were placed on an inverted microscope stage (Nikon Diaphot with Hoffman modulation contrast), and monitored with a video camera and simultaneously video taped. The drop (100 μl) containing the cell was perfused at a rate of 100 μl/min utilizing a peristaltic pump with glass infusion and withdrawal pipettes placed into the drop. Most often, a standard perfusion protocol was employed. The protocol consisted of a 10 min perfusion of the HBS solution, followed by a 10 min perfusion of experimental drug in HBS (ATP or gluconate), followed by a return to the HBS for 10 min, then a 5 min perfusion with a hypotonic HBS solution (280 mOsm/kg water; made hypotonic by lowering the NaCl concentration to 127 mM) and finally a return to HBS for 5 min. Cells that did not continue to meet the morphological criteria described above during the first 10 min of perfusion with HBS were discarded. Cells that did not react to the experimental drug and also did not react to the hypotonic HBS were considered non-reactive and were not included in the final data

(Bobbin et al., 1990). Solutions of ATP and gluconate were made isoosmotic by lowering the concentration of NaCl. All experiments were carried out at room temperature (25°C). Cell length was measured from the video tapes as described (Skellett et al., 1995).

2.2. In vivo procedures

2.2.1. Acute administration of drugs to the cochlea coupled with subsequent long-term survival

Pigmented guinea pigs (250–450 g; 13 animals) of either sex were given atropine to reduce secretions (0.1 mg/kg, s.c.) and then anesthetized by administration of a dose of acetylpromazine (5 mg/kg, i.m.), followed by ketamine (125 mg/kg, i.p.). Additional doses of ketamine (30 mg/kg, i.p.) were given as needed. Under aseptic conditions, the right bulla of each animal was exposed postauricularly and a hole drilled through the bony bulla for visualization of the cochlea. A small (0.3 mm) hole was made in the bony wall of the basal turn of the cochlea slightly apical to the round window for infusion of artificial perilymph (AP). An outflow hole (0.3 mm) to allow the effluent to escape was made in the horizontal semicircular canal wall in the bulla wall. The AP employed in these experiments had a composition of (in mM): 137 NaCl, 5 KCl, 2 $CaCl_2$, 1 NaH_2PO_4, 11 glucose, 12 $NaHCO_3$. Drugs were dissolved in the AP solution just before use, pH was adjusted to 7.4, and the osmotic pressure of the solutions was adjusted (to 300 mOsm/kg water) by lowering the concentration of NaCl. AP containing either ATP (60 mM; 7 animals) or gluconate (60 mM; 5 animals) was infused into the hole in the basal turn scala tympani and out of the hole in the semicircular canal at 2.5 μl/min for 2 h. Following termination of the perfusion, the bulla was closed with sheets of Gelfoam (Upjohn), and the wound closed with interrupted sutures.

Three to four weeks after the above surgery the guinea pigs were anesthetized (urethane, 1.5 g/kg, i.p.), tracheotomized and rectal temperature was maintained at 38 ± 1°C. Additional urethane (0.15 g/kg, i.p.) was administered as required to assure a deep level of anesthesia. The right cochlea was exposed ventrolaterally and both middle ear muscles were sectioned. Electrical potentials were recorded using a silver wire with a balled tip placed on the round window of the cochlea and DPOAEs were recorded from the external ear canal as described below. At the end of the recording procedures the vasculature of the animal was perfused by intracardiac perfusion with 500 ml of phosphate buffered Tyrode solution followed by 500 ml of a mixed aldehyde solution (2.5% glutaraldehyde; 2% paraformaldehyde) in cacodylate buffer at pH 7.2. The bullae were removed, opened and placed in the mixed aldehyde solution where the round window and oval window were opened, a hole was made in the apex of the cochlea and the solution slowly washed through the scala with a pipette. The bullae were kept in the mixed aldehyde solution overnight. Following fixation, the bullae were decalcified using 8% EDTA. Following decalcification, the ears were dehydrated and embedded in glycol methacrylate. Serial 4 μm sections were cut in the plane of the modiolus and stained with toluidine blue.

2.2.2. Cochlear perfusions in acute animal experiments

Acute cochlear perfusion experiments were carried out in urethane anesthetized guinea pigs using methods described previously (Kujawa et al., 1994a). Briefly, the guinea pig's right cochlea was prepared for perfusion by placing holes in the cochlear basal turn perilymph compartment. A hole was placed in scala tympani for the introduction of the perfusates and a hole was placed in scala vestibuli to allow the effluent to escape. The same AP and drug solutions in AP as described above were employed in these experiments. The drug solutions were applied to the cochlea by perfusing the perilymph compartment from basal turn scala tympani to basal turn scala vestibuli at 2.5 μl/min using a micropipette coupled to a syringe pump.

2.2.3. Gross cochlear and auditory nerve potentials

Methods used to monitor the effects of treatments on sound-evoked potentials were similar to those described previously (Kujawa et al., 1994a). The compound action potential of the auditory nerve (CAP amplitude; N_1-P_1), cochlear microphonic (CM) and summating potential (SP) were recorded from the right cochlea utilizing a silver wire (teflon-coated except for the balled tip) placed on the round window and referenced to neck muscle. The evoked responses were amplified (Grass, P15, gain=1000), averaged (over 20 trials) and stored on the computer disk. The responses were evoked by 10 kHz tone pips (0.25 ms rise/fall, 10 ms duration, 200 ms interstimulus interval). Stimuli were computer generated (Tucker-Davis TDT System II hardware), transduced by a speaker and delivered through a hollow ear bar to the right ear of each animal under computer control. Intensity was increased in 6 dB steps over the range 8–92 dB SPL. Custom software was utilized to display and filter the composite waveform (CAP, SP: low pass to 2 kHz; CM: 7.5–15 kHz) for identification of the CAP, CM and SP components across stimulus intensities.

2.2.4. DPOAE generation and measurement

Equilevel primary tones (f_1; f_2; ratio f_2/f_1=1.2) were generated under computer control using Tucker-Davis System II audio processing equipment two separate speakers (Etymotic Research, ER-2) housed within an acoustic probe assembly (see Skellett et al., 1996 for additional details). The acoustic probe assembly was tightly coupled directly to the right ear of each animal.

The cubic DPOAEs ($2f_1-f_2$) and quadratic DPOAEs (f_1-f_2) for f_2=1.5, 1.875, 3, 4.5, 6, 7.5, 9, and 12 kHz were detected by a microphone (Etymotic Research, ER-10) also housed within the probe assembly and amplified using a microphone preamplifier (Etymotic Research, ER-1072) and a dynamic signal analyzer (Hewlett-Packard, 3561A) for fast Fourier transform analysis (averaging 10 discrete spectra) and spectral display (span=1 kHz; BW=3.75 Hz). Intensity functions for the DPOAEs were obtained in response to primaries (range of 20–70 dB SPL) in 5 dB steps. The noise floor associated with this display was approximately −10 to −15 dB SPL.

ATP (disodium salt, cat. no. A-2383) and gluconate (D-gluconic acid, sodium salt, cat. no. G-9005) from Sigma Chemical Company were used in all experiments. As mentioned above, solutions of ATP were made isoosmotic by adjusting the NaCl. This lowered the chloride level but not the sodium level since sodium ATP was used. Lowering the chloride may induce collapse of OHCs (Cecola and Bobbin, 1992). Therefore, sodium gluconate was chosen as a control for both the procedures (acute and chronic in vivo perilymph compartment perfusion and perfusion of the drop) and the lowering of the chloride. In addition, ATP complexes cations and therefore, gluconate which also complexes cations was a control for these effects of ATP (Schubert and Lindenbaum, 1952). The care and use of the animals reported on in this study were approved by the Animal Care and Use Committee of the Medical Center at Louisiana State University.

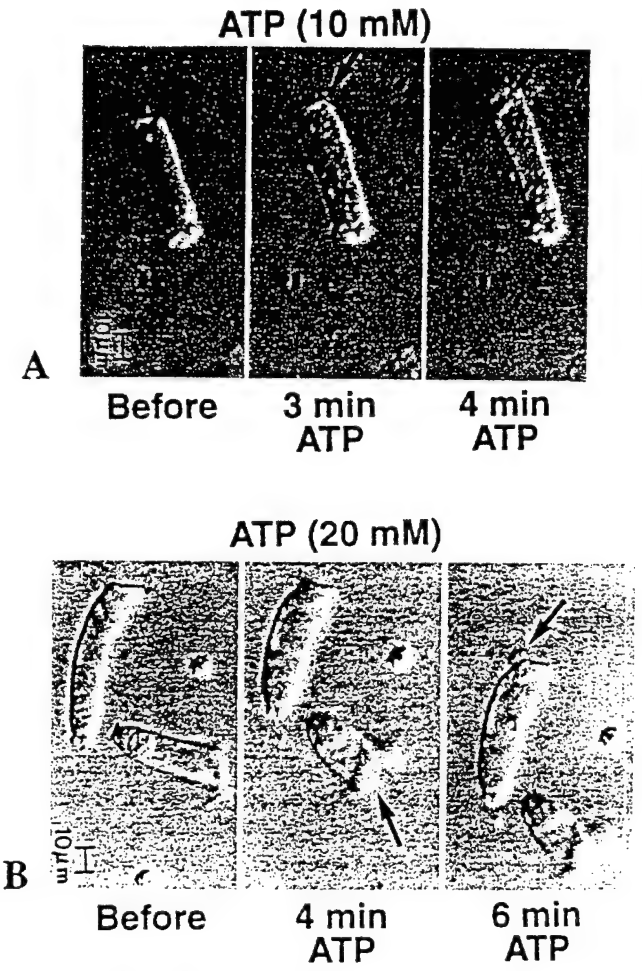

Fig. 1. A: ATP applied at 10 mM resulted in bleb formation in the infracuticular region and eventual bursting of the bleb. B: In different cells, ATP (20 mM) induced a bleb in the shorter OHC before the longer OHC. The bleb burst in the shorter OHC before the bleb in the longer OHC. The shorter cell appears to shorten in response to the application of ATP because the cell lifted off the bottom of the dish.

3. Results

3.1. ATP applied to isolated OHCs

Low concentrations of ATP (1 mM) induced no consistent change in the length or shape of the OHCs. In general, higher concentrations (3–30 mM) of ATP applied to isolated OHCs were found to induce bleb formation, or a swelling of the OHCs, at a point just below the cuticular plate or in the infracuticular region of the cells. In the example shown in Fig. 1A application of 10 mM ATP to the cell resulted in formation of a bleb that eventually burst, releasing the cellular contents and giving the OHC a 'ghost-like' appearance. In additional examples, shown in Fig. 1B, 20 mM ATP initiated formation of a bleb that burst in the shorter cell before initiating bleb formation and bursting in the longer cell. A similar swelling and bleb formation with micromolar concentrations of ATP has been described by Housley et al. (1995) in OHCs in the voltage clamp condition.

To quantitate the incidence of bleb bursting (lysis), the number of OHCs that underwent lysis within the 10 min of ATP exposure were tabulated (Fig. 2). The same concentrations of gluconate induced no bleb formation (1 mM, 10 cells; 3 mM, 8 cells; 10 mM, 12 cells; 20 mM, 18 cells; 30 mM, 13 cells). Cells that leaked their contents and became ghost-like without a visible bleb formation during the 10 min of ATP or gluconate exposure were not included in this quantification (ATP: 3 mM, 1 cell; 10 mM, 3 cells; 20 mM, 1 cell; 30 mM, 8 cells; gluconate: 3 mM, 1 cells; 30 mM, 2 cells). In general, it appears from Fig. 2 that shorter cells were more sensitive to ATP-induced bleb formation with bursting than were longer cells which required higher concentrations. Very long cells (91–110 μm) did not respond (Fig. 2).

3.2. ATP applied to the cochlea: acute effects

Others have demonstrated that ATP applied to the

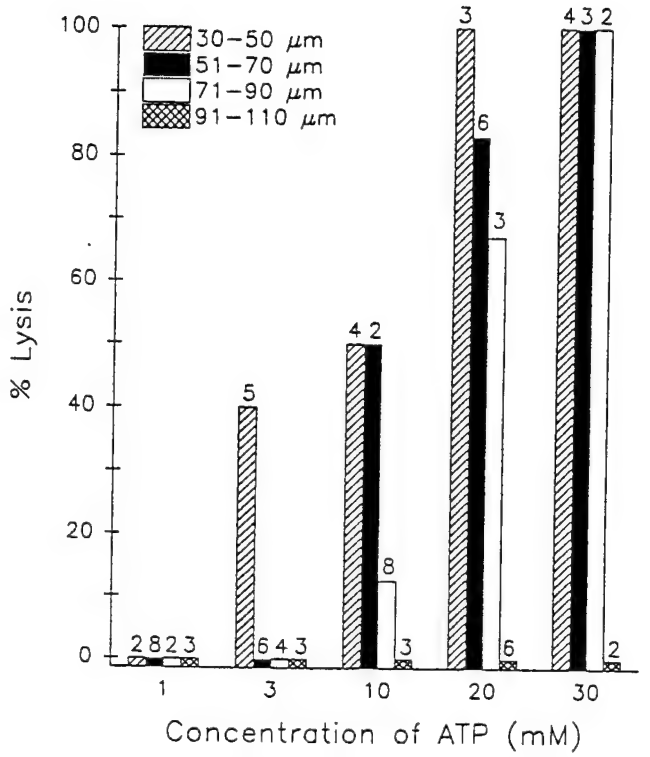

Fig. 2. The relationship between ATP concentration, cell length and bleb bursting (% lysis=number of OHCs where the bleb burst divided by the number of cells tested×100) in OHCs. Numbers above the bars represent the number of cells tested.

perilymph compartment is rapidly broken down by enzymes (Vlajkovic et al., 1996). Previously, we had demonstrated that ATP-γ-S, which is metabolized at a slower rate than ATP, was more potent than ATP in reducing DPOAEs when placed in the perilymph (Kujawa et al., 1994a). Therefore to test for ATP cytotoxicity in vivo, ideally we wanted to use a concentration of ATP that saturated the enzymes responsible for the breakdown process and allowed the ATP to act at the receptors. It was reasoned that the concentration of ATP producing approximately the same response on DPOAEs as ATP-γ-S in the study of Kujawa et al. (1994a) would be an appropriate approximation of this ideal. Preliminary results indicated that 60 mM ATP applied by perilymph compartment perfusion for approximately 2 h decreased the high intensity (70 dB SPL primaries) cubic DPOAE to an extent that was similar to the decrease obtained with 1 mM ATP-γ-S (Fig. 3). Gluconate (60 mM) induced no change (Fig. 3).

3.3. *ATP applied to the cochlea: chronic physiological effects*

Based on the above results, it was assumed that 60 mM was at or above the concentration of ATP necessary to be applied into the perilymph compartment to deliver an effective concentration of unaltered chemical and so test whether ATP kills cells in the cochlea. ATP (60 mM; 7 animals) and gluconate (60 mM; 5 animals) were applied for 2 h through the perilymph compartment. One animal did not recover from the surgery (an ATP animal) and the remaining animals were allowed to recover for 3–4 weeks. Immediately following recovery from the surgical anesthesia, it was observed that the animals receiving the ATP exhibited a head tilt, turning behavior and nystagmus, indicating damage to their vestibular systems. After the 3–4 week recovery interval, the animals were prepared as described in Section 2 for recording of cochlear potentials and DPOAEs. The middle ear of only one animal was abnormal, being filled with purulent material and so it was discarded (a gluconate animal). Recordings from the ATP animals (n=6) revealed the complete absence of cochlear potentials in response to tone bursts of 10 kHz compared to good average responses from the gluconate animals (n=4; Fig. 4). In addition, all cubic and quadratic DPOAEs were at the noise floor in the ATP animals compared to the good average values from the gluconate animals (only a representative frequency of cubic DPOAEs is shown in Fig. 4).

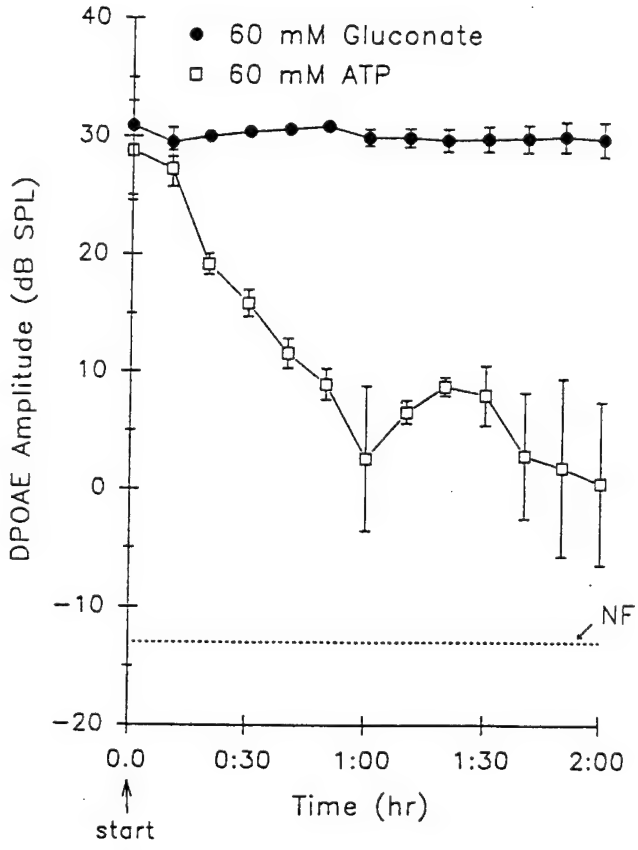

Fig. 3. Magnitude (mean ± S.E.) of the cubic DPOAE recorded in response to 70 dB SPL primaries (L_1=L_2; f_1=6.25 kHz, f_2=7.5 kHz, $2f_1-f_2$=5 kHz) during the perfusion of the perilymph compartment of the cochlea with gluconate (60 mM; n=3) and ATP (60 mM; n=3) in acute animals. Start indicates the start of the perfusions (0 h). NF is the representative average noise floor.

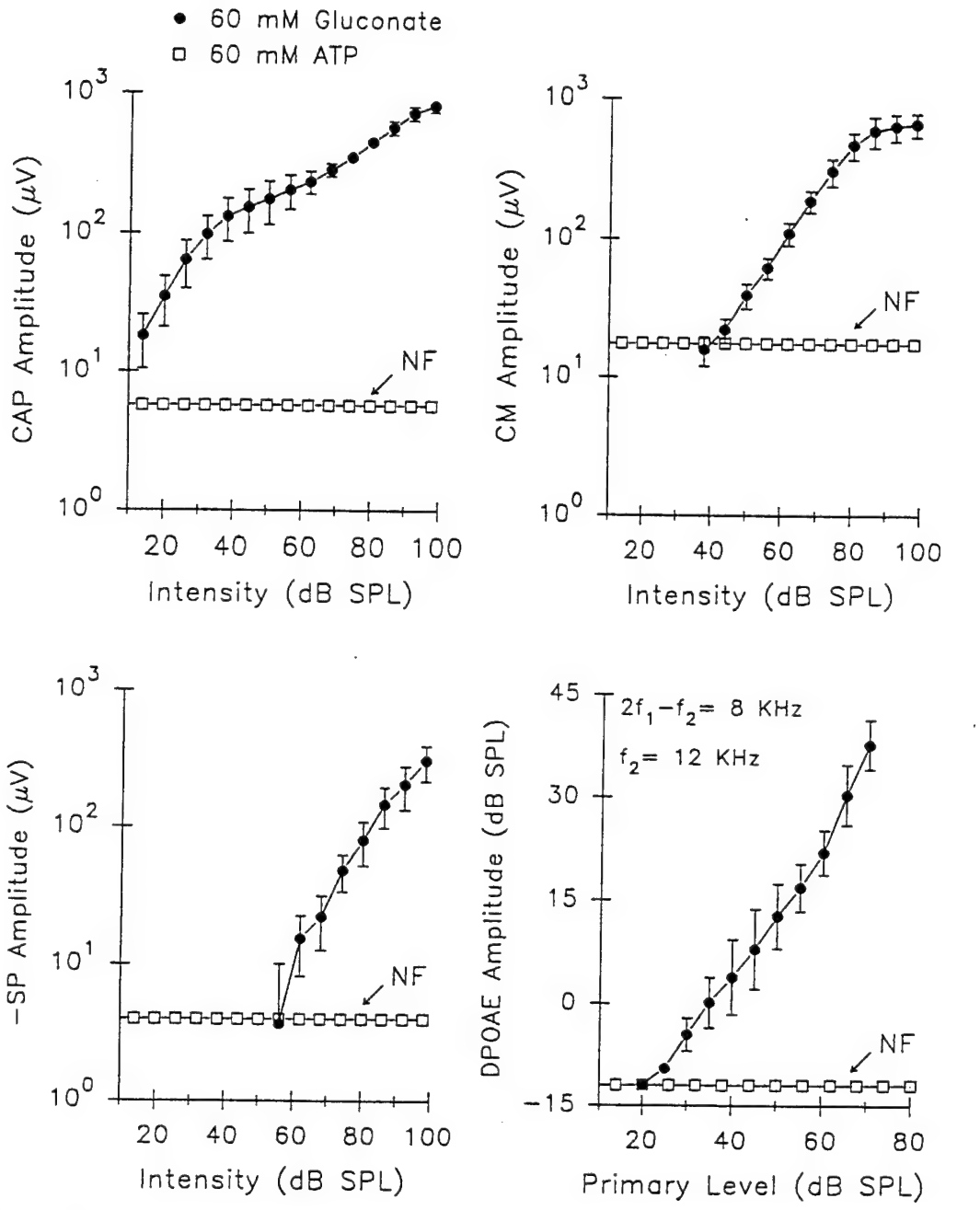

Fig. 4. Effect (means ± S.E.) of ATP (60 mM; n=6) and gluconate (60 mM; n=4) perfused through the perilymph compartment for 2 h followed by a recovery period of 3–4 weeks on CAP, SP and CM recorded from the round window and the cubic distortion product ($2f_1-f_2$=8 kHz) recorded from the ear canal. NF is the representative average noise floor.

3.4. *ATP applied to the cochlea: chronic anatomical effects*

Cochleas from four ATP and four gluconate animals, that were examined for cochlear potentials and DPOAE above, were subsequently prepared for histology. In general, both cochleas of the gluconate animals (right ear perfused with gluconate; left ear untreated) and the left cochlea (untreated) of the ATP animals were normal except for changes which were attributed to histological preparation (Fig. 5A,B). In contrast, the cells in the organ of Corti and stria vascularis of ATP perfused cochlea were almost completely destroyed (Fig. 5C,D). A section through a third turn of a typical ATP-treated cochlea is shown in Fig. 5C,D. In this example all of the cells of the organ of Corti and the stria vascularis are absent, and the tectorial membrane appears to be rolled up in scala media. Spiral ganglion neurons are obviously reduced in number (Fig. 5C). In addition, although scala media appears empty with some hydrops possibly present, the scala vestibuli and tympani are filled with proliferating connective tissue

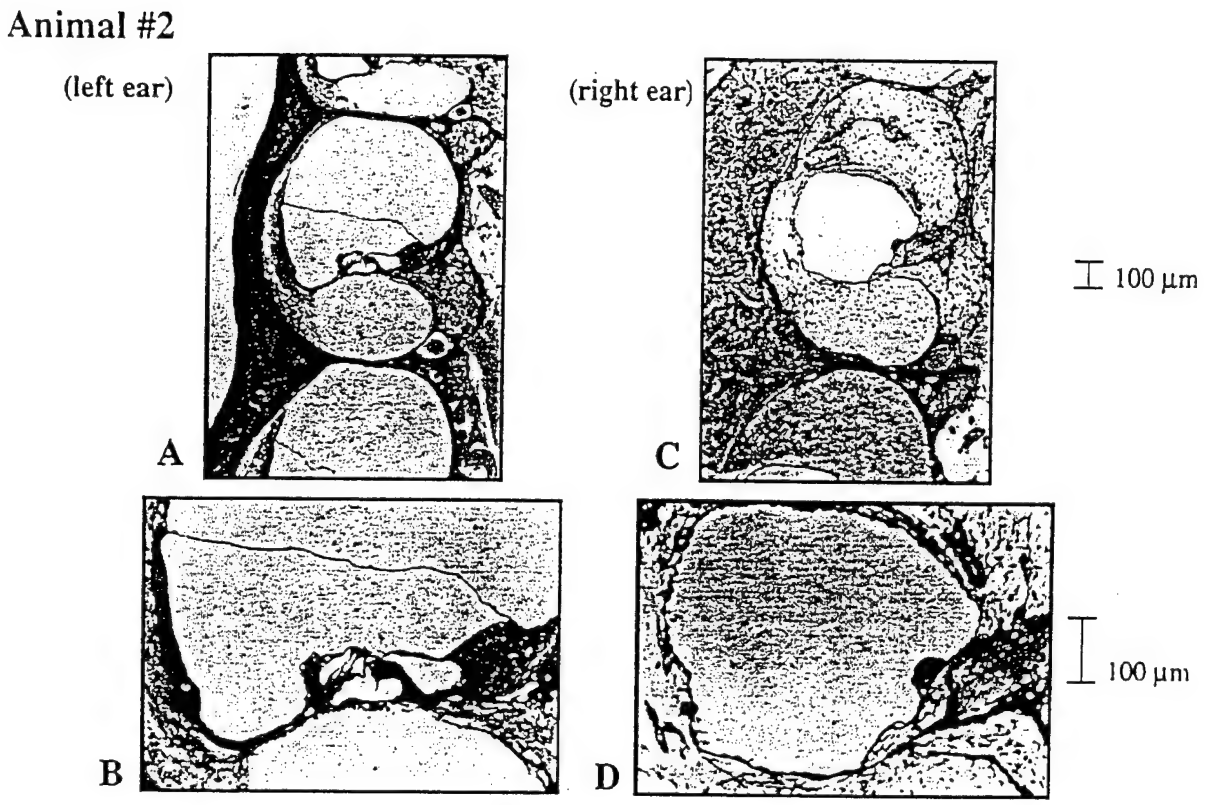

Fig. 5. Approximate mid-modiolar sections showing a third turn of a normal left cochlea (A, B) and an ATP (60 mM)-treated right cochlea (C, D) of an animal whose cochlear potential and DPOAE data are included in Fig. 4.

(Fig. 5C,D). In all four ATP-treated cochlea, the scala tympani and scala vestibuli of the upper, apical turns were filled with proliferating connective tissue to a greater extent than were more basal turns. The three other ATP-treated cochlea appeared the same as the cochlea shown in Fig. 5C,D, except for one which had some supporting cells in the organ of Corti and no obvious reduction in the number of spiral ganglion neurons. Although vestibular tissue was not the focus of the study, histological sections through the vestibular labyrinth of all four ATP-treated animals indicated damage similar to that observed in the cochlea (i.e. cells absent together with proliferation of connective tissue).

4. Discussion

Results demonstrate that ATP may act as a cytotoxin and mitogen in the cochlea. The study was based on the results of Housley et al. (1995) who documented a large increase in OHC volume that was initiated in the infracuticular region of the cell due to the application of micromolar ATP to voltage clamped OHCs. In the present study, when ATP was applied to isolated OHCs that were not voltage or current clamped, the same phenomenon was observed but at millimolar concentrations and not the micromolar concentrations reported by Housley et al. (1995). In addition, when ATP (60 mM) was applied into the cochlea and examined 3–4 weeks after the application to test ATP for cytotoxicity, cochlear potentials and DPOAEs were absent and almost all of the cells in the cochlea were found to be destroyed with the exception of a proliferation of connective tissue. The latter effect indicates that ATP had a mitogenic effect on the fibrocytes in the cochlea. The results indicate that ATP administration to the perilymph triggered a complex series of events. Whether the cell death obtained in vivo is due to the same type of necrosis-like event observed in isolated OHCs remains to be determined.

The reason that higher concentrations of ATP were necessary to observe bursting of isolated OHCs in the present study than were necessary in voltage clamped OHCs in the study by Housley et al. (1995) may be complex. In voltage clamped OHCs, ATP in micromolar amounts induces more cation current in short OHCs than in longer ones, presumably because of a lower ATP receptor density on the longer OHCs (Housley et al., 1995; Chen et al., 1995a). This relationship was similar to the one found in the present study, in that, short OHCs were more sensitive to ATP-induced cell lysis, possibly indicating that the cell lysis may be a reflection of the greater amount of cation current and so receptor density in the shorter OHCs. If the ATP was acting on ionotropic ATP receptors on the OHCs in both clamped and non-clamped OHCs, then why did

non-clamped OHCs require a thousand fold greater concentration of ATP to induced lysis than voltage clamped OHCs? We know that isolated OHCs have accumulated ions such as Na^+ and Ca^{2+} bringing their membrane potentials towards zero (Erostegui et al., 1994; Ikeda et al., 1992). The reversal potential for ATP induced ion flux (Na^+ and Ca^{2+}) is near zero (Chen et al., 1995a,b; Nakagawa et al., 1990). Therefore, since the isolated OHCs' membrane potential is close to zero, there is little if any chemical or ionic gradient for Na^+ and Ca^{2+} flux (and water) through the ligand gated ion channel complex opened upon application of ATP. Under whole cell voltage clamp, the cells are dialyzed with the pipette solution which lowers the Na^+ and Ca^{2+} concentrations, and re-establishes the gradients. Thus, one reasonable hypothesis for millimolar concentrations of ATP being required to induce bleb formation in non-patch clamped cells is that the gradient for ATP induced ion flux in these isolated OHCs is less than it is in OHCs under the whole cell voltage clamp condition.

The type of swelling and bursting observed with ATP on the isolated OHCs is unusual, although a similar bursting just below the cuticular plate has been reported to be induced by triethyltin (Clerici et al., 1993). The most common type of OHC swelling observed is a total cell shortening and increase in width that leads to a rounding of the cell with bursting usually occurring adjacent to the nucleus (Goldstein and Mizukoshi, 1967). The latter is observed in response to high concentrations of potassium, and hypotonic solutions (Cecola and Bobbin, 1992; Crist et al., 1993; Goldstein and Mizukoshi, 1967; Skellett et al., 1995). Housley et al. (1995) suggest that the ionotropic ATP receptors on OHCs are located near the stereocilia. One would expect then that opening of an ion channel at the stereocilia end of the cell would result in an increase in ions and water in the whole cell and the whole cell would swell and burst. Since this did not occur either in our experiments or in the experiments reported by Housley et al. (1995), the bleb and bursting in response to ATP at the infracuticular region may indicate a separate micro compartment that is not continuous with the rest of the OHC.

The sites and mechanisms responsible for the destruction of the cells in the cochlea and vestibular labyrinth in the post 60 mM ATP-treated chronic animals is unknown. It appears not to be an infection since the histology was not typical of an infectious process. In addition, the type of cellular reaction observed in the cochlea of every ATP animal was not observed in any of the gluconate animals. One speculation is that the ATP acted on ionotropic P2X receptors on cells in the cochlea and in the vestibular labyrinth (Aubert et al., 1994). At these receptors, ATP initiated necrosis by inducing a large influx of cations into the cells that resulted in swelling and bursting as observed in the isolated OHCs and as described in other cells (Tatham and Lindau, 1990; Steinberg and Silverstein, 1989). Evidence that the ATP acted on receptors will have to be obtained in the future by testing whether ATP antagonists will block cell death. Non-receptor mechanisms may also have induced the cell death. For instance, the ATP may have complexed cations such as calcium and magnesium (Wilson and Chin, 1991) and induced such low cation concentrations that the cells died. However, the control, gluconate, also complexes cations (Schubert and Lindenbaum, 1952) and it demonstrated no toxicity. In addition, given the large amount of ATP degradation that occurs when ATP is instilled into perilymph, the toxic chemical may be a degradation product of the ATP, such as adenosine, adenosine 5′-diphosphate, adenosine 5′-monophosphate or phosphate (Vlajkovic et al., 1996). Arguing against a non-receptor, non-cellular specific mechanism is the apparent mitogenic response of the connective tissue. Finally, the concentration of ATP tested (i.e. 60 mM) may be far above the threshold concentration necessary to induce cell death in the cochlea. Future experiments will test lower concentrations of ATP and ATP-γ-S, a derivative that is hydrolyzed at a much slower rate than ATP.

Whether the cytotoxicity of ATP in the cochlea demonstrated in the present study has any functional significance is unknown. Speculation may include functions proposed in other systems. For example, it has been proposed that ATP receptors such as those existing in the cochlea may act as 'suicide receptors' (Falzoni et al., 1995; Di Virgilio et al., 1990; Beyer and Steinberg, 1991). In general, when cells are infected with a virus, cytotoxic T lymphocytes invade the tissue and release various cytotoxic chemicals including ATP which kill the infected cells (Falzoni et al., 1995). In the cochlea, the ATP receptors may be there to interact with the ATP released from the lymphocytes resulting in the death of the virus infected cells, as observed by Keithley et al. (1989), Woolf et al. (1988) and Schuknecht (1993; p. 239). ATP may also play a role in the destruction of cells in the cochlea following intense noise exposure (Chen et al., 1995a). If such is the case then drugs that block ATP receptors may be useful in protecting the cells from death during noise exposure or viral infection. Alternatively, as suggested by Falzoni et al. (1995), the cytotoxicity may be a pharmacological event having nothing to do with the physiological function of the receptors. Instead the receptors may function to enhance intercellular communication between cells that differentiated into multicellular structures (Falzoni et al., 1995).

Schuknecht (1993, p. 239 in Fig. 5.88) described proliferation of connective tissue into the perilymph compartment of a virus infected human cochlea that was similar to that described here. Eight fibrocyte cell types

have been described to be normally present in the spiral ligament of the cochlea (Spicer and Schulte, 1996). Others have demonstrated that extracellular ATP acting on metabotropic ATP receptors (P2Y) has a mitogenic effect on fibrocytes (e.g. Huang et al., 1993; Wang et al., 1994). Thus it is possible that application of ATP to the perilymph compartment may have stimulated the division of existing fibrocytes in the cochlea which resulted in the proliferation of connective tissue observed in the perilymph scala. Whether ATP released during virus infection induces fibrosis in the human cochlea remains to be demonstrated.

In summary, results indicate that ATP is cytotoxic to isolated OHCs. In addition, when placed into the perilymph compartment, ATP killed a majority of the cells in the cochlea while possibly stimulating fibrocyte proliferation. The mechanisms involved are unknown and may involve ATP receptors. We speculate that the functional significance of the cytotoxicity may lie in the deafness observed subsequent to intense noise exposure or viral infection of the cochlea.

Acknowledgments

Thanks to Latasha Bright for technical assistance and to Douglas Webster, Ph.D. and Sandy Blanchard for carrying out the histology. Thanks to Bradley A. Schulte, Ph.D. for evaluating the histology of the cochlea and identifying the proliferation of the connective tissue. Thanks to Charles H. Norris, Ph.D. for suggesting the effluent port be made in the horizontal semicircular canal in the chronic animals and evaluating the histology of the vestibular labyrinth. This work was supported in part by Research Grants P01-DC00379 and R01-DC00722 from the National Institute on Deafness and Other Communication Disorders, National Institutes of Health, and DAMD 17-93-V-3013, Kam's Fund for Hearing Research, and the Louisiana Lions Eye Foundation.

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Submitted Hearing Research 1997

Additional pharmacological evidence that endogenous ATP modulates cochlear mechanics.

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Abstract

In the cochlea, outer hair cells (OHCs) generate the active cochlear mechanics (OHCs) whereas the supporting cells, such as Deiters' cells and Hensen's cells may play a role in both the active and passive cochlear mechanics. The presence of ATP receptors on these cells indicates that endogenous ATP may have a role in cochlear mechanics. To explore this possibility, the effects of the ATP antagonist, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), were studied both in vitro on isolated OHCs, Deiters' cells, Hensen's cells and pillar cells using the whole-cell configuration of the patch-clamp technique and in vivo on sound evoked cochlear potentials (cochlear microphonic, CM; summating potential, SP; compound action potential , CAP) and distortion product otoacoustic emissions (DPOAEs) using cochlear perilymphatic perfusion. Results show that PPADS (100 μM) reduced the inward current evoked by 5-10 μM ATP in OHCs, Deiters' cells, Hensen's cells and pillar cells. This effect of PPADS was slow in onset and was slowly reversed to a varying degree in the different cell types. In vivo application of PPADS in increasing concentrations reduced the sound evoked CAP, SP and increased N1 latency starting at about 0.33 mM (SP) and 1mM (CAP and N1 latency). PPADS (0.33 to 1 mM) reversibly suppressed the initial value of the quadratic DPOAE and reversed the 'slow-decline' in the quadratic DPOAE that occurs during continuous stimulation with moderate level primaries. These results, together with the similar effects of the ATP antagonist suramin reported previously (Skellett et al., 1997), may be evidence that endogenous ATP acting on cells in the organ of Corti alters cochlear mechanics.

Key words: pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid; PPADS; ion channels; otoacoustic emissions; Deiters' cells; pillar cells; outer hair cells; Hensen's cell

1. Introduction

Adenosine triphosphate (ATP) has been suggested to serve as a neurotransmitter, neuromodulator, cytotoxin and mitogen in various systems including the cochlea (see: Bobbin, 1996; Bobbin et al., 1997a; Bobbin et al., 1997b; Burnstock, 1990; Eybalin, 1993). Bobbin and Thompson (1978) first suggested ATP as a candidate for a neurotransmitter in the cochlea based on its relative potency in reducing the compound action potential of the auditory nerve(CAP). Kujawa et al. (1994a) demonstrated that ATP-γ-S, an analogue of ATP that is not rapidly metabolized, was active at ionotropic ATP receptors on OHCs and it also was one of the most potent compounds studied in affecting cochlear potentials when instilled into the perilymph compartment of guinea pig cochleas. Among the many effects of ATP-γ-S in the cochlea was that it abolished cubic distortion product otoacoustic emissions (DPOAEs). Active and passive mechanical transduction processes in the cochlea generate DPOAEs (Bobbin, 1996; Brownell, 1996; Frank and Kossl, 1996). Therefore, the suppression of the DPOAEs by ATP-γ-S suggests that activation of ATP receptors in the cochlea has powerful effects on cochlear mechanics.

One criterion in proving that a physiological response is due to a given substance, is that antagonists of the substance will block the physiological response (e.g., Bobbin et al., 1985). Few studies have examined the effects of ATP antagonists on cochlear function. Kujawa et al. (1994b) demonstrated that the ATP antagonist cibacron blue (Collo et al., 1996; Burnstock and Warland, 1987) abolished the cubic DPOAE. In contrast suramin, another ATP antagonist, had no effect on the cubic DPOAE, although it did reduce the amplitude of the summating potential (SP) and the CAP (Kujawa et al., 1994b). Some of the results with cibacron were recently demonstrated to be probably due to the effects of the drug on voltage activated ion channels in OHCs and Deiters' cells (Skellett et al., 1997). Suramin had no effect on these channels (Skellett et al., 1997).

In contrast to its lack of effect on the cubic DPOAE, suramin reversibly suppressed the 'slow-decline' in amplitude of the quadratic DPOAE that occurs in response to continuous primary presentation (Skellett et al., 1997). In addition, suramin shifted the intensity function of the quadratic DPOAE to the left, indicating an increased magnitude at a given dB SPL of the primaries. It was speculated that this data indicated that endogenous release of ATP, acting on ATP receptors on Deiters' cells, may underlie this 'slow-decline' in amplitude and that the slow decline was a reflection of the effects of endogenous ATP on cochlear mechanics (Skellett et al., 1997). However, one must be circumspect in attributing the effect of suramin on the quadratic DPOAE to blockade of endogenous ATP as suramin has been reported to have many effects other than blockade of ATP at ATP receptors. For instance, suramin has been reported to inhibit the ectonucleotidases (Bailey and Hourani, 1994; Ziganshin et al., 1995), diacylglycerol kinase (Kopp and Pfeiffer, 1990) and several protein tyrosine phosphatases (Ghosh and Miller, 1993). Further evidence as to the mechanism by which suramin affects the quadratic DPOAE may only be obtained by examining the effects of additional ATP antagonists on this phenomena. Therefore, one purpose of the present study was to test whether pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), a more potent ATP antagonist than suramin at some ATP ionotropic receptors (Collo et al., 1996; Ziganshin et al., 1995), would exhibit pharmacological effects similar to those of suramin on DPOAEs and cochlear potentials. In addition, the pharmacology of PPADS on ATP induced currents and on voltage gated ion currents at the level of the single cells in the organ of Corti were explored. Preliminary results have been presented (Bobbin et al., 1997a).

2. Methods

2.1 In Vitro Procedures

2.1.1 Isolation of cells from the organ of Corti

OHCs, Deiters' cells, Hensen's cells and pillar cells from pigmented guinea pigs were isolated as described previously (Chen et al., 1995a,b). The cells were identified by well known characteristic anatomical features. Animals were anesthetized with pentobarbital (35 mg/kg, i.p.), decapitated, and the bulla separated and placed in a modified Hank's balanced saline (HBS). The bone surrounding the cochlea was removed, and the organ of Corti was placed in 200 µl of HBS containing collagenase (1 mg/ml, Type IV, Sigma) for 5 min. The cells were then isolated and transferred into the dishes containing a 150 µl drop of HBS using a microsyringe and stored at room temperature.

2.1.2 Whole-cell voltage clamp

Single dispersed guinea pig OHCs, Deiters' cells, Hensen's cell as well as pillar cells were voltage clamped using the whole-cell variant of the patch-clamp technique (Hamill et al., 1981) with Axopatch 200A patch-clamp amplifiers (Axon Instruments). Patch electrodes were fabricated from borosilicated capillary tubing (Longreach Scientific Resources) using a micropipette puller (Sutter Instrument Co.), and fire polished on a microforge (Narashige Scientific Instrument Lab.) prior to use. Membrane currents were filtered at 5 kHz (- 3dB) using a four-pole low-pass Bessel filter digitized with a 12-bit A/D converter (DMA Interface, Axon Instruments), and stored for off-line analysis using a pentium microcomputer. Voltage paradigms were generated from a 12-bit D/A converter (DMA Interface, Axon Instruments) using pClamp software (Axon Instruments). After establishment of the whole-cell configuration, series resistance and cell capacitance compensation were carried out prior to recording with 80 % series

resistance compensation normally applied. No subtraction of leakage current was made.

2.1.3 Solutions

The HBS utilized for isolating cells and perfusing the bath contained (in mM): NaCl, 145; KCl, 5.4; $CaCl_2$, 2.5; $MgCl_2$, 0.5; HEPES, 10; and glucose, 10. The HBS solution was adjusted to a pH of 7.40 with NaOH and to 300 mOsm/kg H_2O with sucrose. The K^+ internal solution contained (in mM): KCl, 140; $MgCl_2$, 0.5; HEPES, 5; EGTA, 11; $CaCl_2$, 0.1; Na_2ATP, 2; and Na_2GTP, 0.1. The internal solution was adjusted to a pH of 7.35 with HCl and had an osmolality of 284 mOsm/kg H_2O adjusted with sucrose. The drugs tested were freshly prepared at desired concentrations in the HBS external solution. All the drug solutions were delivered from a U-tubing system as described previously while the bath was continuously exchanged with a perfusion system separate from the U-tubing system (Chen et al., 1995a,b). All experiments were conducted at room temperature (22~24°C).

2.2 In vivo Procedures.

2.2.1 Subjects

Experiments were performed on pigmented guinea pigs of either sex weighing between 250 and 400 g. Anesthetized animals (urethane, Sigma; 1.5 g/kg, i.p.) were tracheotomized and were allowed to breath unassisted. ECG and rectal temperature were monitored throughout each experiment and temperature was maintained at 38 ± 1°C by a heating pad. Additional urethane was administered as required to maintain an adequate depth of anesthesia. Surgical procedures have been described previously (Kujawa et al., 1994a,b). In all animals the right auditory bulla was exposed using a ventrolateral approach and tendons of the right middle ear muscles were sectioned.

2.2.2 Cochlear perfusion experiments

Perfusions were carried out using methods described previously (Kujawa et al., 1994a,b). The artificial perilymph (AP) had a composition of (in mM): NaCl, 137; KCl, 5; $CaCl_2$, 2; NaH_2PO_4, 1; $MgCl_2$, 1; glucose, 11; $NaHCO_3$, 12. The PPADS was mixed with the AP on the day of use at desired concentrations. The pH of all solutions was adjusted to 7.4 when necessary. Perfusates were introduced into the cochlear perilymph at room temperature and at a rate of 2.5 μl/min for 15 min through a hole in basal turn scala tympani and were allowed to flow from the cochlea through an effluent hole placed in basal turn scala vestibuli. Effluent was absorbed within the bulla using small cotton wicks. In all animals, the first two perfusions were of AP alone. These perfusions were performed to achieve a stable baseline to which subsequent alterations in the artificial perilymph and drug-related changes could be compared. These perfusions were followed by perfusions of the AP or experimental drug.

2.2.3 Gross cochlear and auditory nerve potentials

Methods used to monitor the effects of multiple perilymphatic perfusions on sound-evoked potentials were similar to those described previously (Kujawa et al., 1994a, 1994b). The compound action potential of the auditory nerve (CAP amplitude; N_1-P_1), N_1 latency, cochlear microphonic (CM) and summating potential (SP) were recorded from the right cochlea utilizing a silver wire (teflon-coated except for the tip) placed in basal turn scala vestibuli. The evoked responses were amplified (Grass, P15, gain = 1000), averaged (over 20 trials) and stored on the computer disk. The responses were evoked by 10 kHz tone pips (0.25 ms rise/fall, 10 ms duration, 200 ms interstimulus interval). Stimuli were computer generated (Tucker-Davis TDT System II hardware), transduced by a speaker and delivered through a hollow earbar to the right ear of each animal under computer control. Intensity was increased in 6 dB steps over the range 8

- 92 dB SPL. Custom software was utilized to display and filter the composite waveform (CAP, N_1 latency, SP: low pass to 2 kHz; CM: 7.5 - 15 kHz) for identification of CAP threshold and amplitudes of the CAP, CM and SP components and N_1 latency across stimulus intensities.

2.2.4 DPOAE: Stimulus generation and response monitoring

The instrumentation employed in these experiments has been described (Skellett et al., 1997). Briefly, quadratic (f_2-f_1 = 1.25 kHz) and cubic ($2f_1$-f_2 = 5 kHz) DPOAEs were elicited by equilevel primary stimuli (f_1 = 6.25kHz; f_2 = 7.5 kHz) sent to two separate speakers housed within an acoustic probe assembly. The acoustic probe assembly was tightly coupled directly to the right ear of each animal. The DPOAEs were detected by a microphone also housed within the probe assembly and amplified using a microphone preamplifier and sent to a dynamic signal analyzer for fast Fourier transform analysis (averaging 10 discrete spectra) and spectral display (span = 1 kHz; CF = DP frequency; BW = 3.75 Hz). The noise floors associated with these display windows averaged approximately -15 dB SPL for the f_2-f_1 DPOAE and -18 dB SPL for the $2f_1$-f_2 DPOAE when measured at points ±50 Hz from the DPOAE frequency.

As described previously (Kujawa et al., 1995, 1996), continuous, moderate-level, primary stimulation (f_1 = 6.25 kHz, f_2 = 7.5 kHz, L_1 = L2 = 60 dB SPL) generated a standard pattern of time-varying alterations in the amplitude of the quadratic (f_2-f_1 = 1.25 kHz) DPOAE. One hundred consecutive 10-spectra averages of distortion product amplitude were obtained during continuous primary stimulation. Each of these averages required approximately 5s to complete for a total of 500 s (8.3 min) of stimulation. The primary tones were then simultaneously turned off and there was a 1 min rest from primary stimulation. Following this rest, the primaries were re-introduced and 40 consecutive 10-spectra averages of distortion product amplitude were obtained (total time approximately 200 s or 3.3 min of stimulation).

At least a 15 min period of rest from primary stimulation separated each test condition from the next. This period was usually the time (15 min) during which the cochlea was perfused. Immediately (within 2 min), after the perfusion was terminated, the primaries were turned on (60 dB SPL) and recording of the time-varying amplitude of the quadratic DPOAE commenced. Following this recording, without any intervening rest, the level of the primaries was increased from 60 to 70 dB SPL and the amplitude growth functions for the quadratic DPOAE obtained by decreasing the intensity of the primaries in 5 dB steps until the noise floor was reached. This was immediately followed by the same procedure (descending primary intensity) to obtain the amplitude growth functions of the cubic DPOAE.

Effects of treatments were quantified using repeated measures analysis of variance (ANOVA) and Student Newman Keuls multiple range test. Pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) was purchased from Research Biochemicals International (Cat. No. P-178) and was used the same day that it was placed into solution. The ATP was purchased from Sigma. The care and use of the animals reported on in this study were approved by LSUMC's Institutional Animal Care and Use Committee.

3. Results

3.1. Effects of PPADS on ATP- and voltage-evoked responses in OHCs, Deiters' cells, Hensen's cells and pillar cells.

As described previously (e.g., Chen et al., 1995a,b, 1997; Skellett et al., 1997; Sugasawa et al.,1996b) ATP evoked inward currents at the holding potential (Vh) in OHCs (Vh = -60 mV), Deiters' cells (Vh = -80 mV) and Hensen's cells (-80 mV). What has not been described to date is the large ATP (5 μM) evoked inward currents at the holding potential (Vh = -80 mV) in pillar

cells that we observed and report here.

PPADS (100 μM) reduced the ATP (10 μM) induced peak current in OHCs by 8 ± 7 % (n = 9) compared to a pre-PPADS ATP control when ATP and PPADS were co-applied for 10 s by the U-tubing system (Fig. 1). However, following a 1 min wash of the bath after the co-application of ATP and PPADS, ATP-induced current was decreased by 53 ± 7 % (n = 8) compared to control, indicating that PPADS may be an open channel blocker or the onset of the blocking effect of PPADS may be slow as reported in other systems (Collo et al., 1996). In addition, the blocking effect of PPADS was not reversed with continued washing of the bath during the time (10 min) that the OHCs could be successfully voltage clamped without deterioration of the seal. Thus the ATP-induced response was still reduced by 59 ± 10 % (n = 4) 10 min after the co-application of ATP and PPADS and continous washing of the bath. To test whether PPADS is an open channel blocker, PPADS (100 μM) was applied alone for 10 s by the U-tube system (2 to 3 min after a control ATP application). Following a 1 min wash out of the PPADS, the ATP (10 μM)-induced response was completely blocked (98 ± 2 %; n = 4), suggesting that PPADS is not an open channel blocker (Fig. 1). PPADS was also examined for an affect on the voltage-gated currents in OHCs. As shown in Fig. 2, PPADS (100 μM) had no significant effect on the whole cell current (n = 4).

The effect of PPADS on ATP-gated current was also tested in Deiters' cells. As illustrated in Fig. 3, a co-application of ATP (5 μM) and PPADS (100 μM) for 10 s by way of the U-tube did not decrease the ATP-gated peak current but rather increased it by 7 ± 13 % (n = 14) compared to a pre-PPADS ATP control. However, the current induced during the ATP/PPADS application decayed much faster compared to the control ATP-induced current (Fig. 3). The ATP-induced current was reduced by 94 ± 1 % (n = 14) when the ATP was applied 1 min after washout of the co-applied ATP and PPADS (Fig. 3). Similar to the effect of PPADS in OHCs,

this blocking effect of PPADS in Deiters' cells was slow to reverse with washing. The ATP-induced response was still blocked by 83 ±4 % (n = 6) after 10 min of washing (Fig. 3) and 63 ± 5 % (n = 4) after 20 min of washing. When PPADS (100 μM) was applied alone to the Deiters' cells for 10 s by the U-tube, the ATP (5 μM)-induced current was almost completely blocked (93 ± 3 %; n = 7) 1 min after the application of PPADS, further indicating that the onset effect of PPADS was slow and PPADS is not an open channel blocker (Fig. 3). The effect of PPADS on voltage-gated currents in Deiters' cells was also monitored. As indicated in Fig. 4, 100 μM PPADS did not significantly affect the whole cell current in Deiters' cells (n = 5).

The effect of PPADS on ATP-gated current in another type of supporting cell, Hensen's cells, was also examined. The ATP-induced current response in Hensen's cells was different from that in Deiters' cells. An application of ATP (5 μM) induced a biphasic inward current in Hensen's cells (Fig. 5). This response is similar to that observed by others in these cells (Sugasawa et al., 1996b). In the Hensen's cells, PPADS increased the ATP-induced peak current by 47 ± 21 % (n = 10) when the ATP (5 μM) and PPADS (100 μM) were co-applied by the U-tube for 10 s compared to a control ATP application (Fig. 5). However, similar to Deiter's cells, the current induced during the ATP/PPADS application decayed much faster compared to the control ATP-induced current (Fig. 5). Similar to the effects in OHCs and Deiters' cells, the ATP-induced current was reduced by 92 ±2 % (n = 9) when applied following a 1 min washout of the ATP/PPADS combination (Fig. 5). In contrast to all of the other cells tested, the blocking effect of PPADS in Hensen's cells was reversed to the greatest extent with continuous washing of the bath (Fig. 5). The ATP-induced current was reduced by only 47 ± 13 % (n = 8) after 10 min of wash and by only 11 ± 17 % (n = 8) after 20 min of wash out of the co-applied ATP and PPADS. One min after the application of PPADS (100 μM) alone for 10s by the U-tube, the ATP (5 μM)-induced response was blocked (92 ± 5 %; n = 5; Fig. 5). In addition, as shown in Fig. 6, PPADS

did not have a detectable effect on the whole cell current in Hensen's cells (n = 4).

An ATP-gated current response was also discovered in pillar cells, another type of supporting cell in the organ of Corti. A large inward current was induced at holding potential of -80 mV with the application of ATP (5 μM) for 10 s by the U-tube to pillar cells (Fig. 7). A co-application of ATP (5 μM) with PPADS (100 μM) by the U-tube for 10 s resulted in an increase in the peak current by 30 ± 21 % (n = 5) compared to an ATP control (Fig. 7). However, similar to Deiter's cells and Hensen's cells, the current induced during the ATP/PPADS application decayed much faster compared to the control ATP-induced current (Fig. 7). Similar to the effect observed in OHCs, Deiters' cell and Hensen's cells, the ATP-induced current in pillar cells was blocked (86 ± 6 %; n = 5) following a 1 min washout of the drug combination (Fig. 7). The degree of reversal of the blocking effect of PPADS in pillar cells varied greatly with continuous washing of the bath (Fig. 7). The ATP-induced current was reduced by only 47 ± 32 % (n = 4) after 10 min of wash and by only 50 ± 28 % (n = 4) after 20 min of wash out of the co-applied ATP and PPADS. One min after the application of PPADS (100 μM) alone for 10 s by the U-tube, the ATP (5 μM)-induced current was blocked (96 ± 2 %; n = 3). In addition, PPADS (100 μM) did not change the whole cell current in pillar cells (n = 3; Fig. 8).

3.2. Effects of PPADS on cochlear potentials

A standard perfusion protocol similar to that previously described was employed (Kujawa et al., 1994a; 1996). First, two or more 15 min perfusions of artificial perilymph (AP) alone were performed. These were followed by successive 15 min perfusions with increasing concentrations of drug. Then AP was used to wash (15 min) the drug from the perilymphatic space. In general, responses measured following the AP perfusion just before drug served as the pre-drug post perfusion baseline to which all drug-related changes recorded immediately following drug

perfusions were compared. Measurements were made within 2 min of terminating the perfusions. Because we have published the lack of effect of multiple perfusions of this artificial perilymph on the cochlear potentials previously (Kujawa et al., 1994a), only one multiple artificial perilymph perfusion was carried out to confirm that the vehicle itself induced no change.

PPADS was tested on the intensity functions of CAP, SP, CM and the N_1 latency (n = 5 animals). Multiple 15 min perfusions of PPADS in a cumulative manner (0.033, 0.10, 0.33, 1.0 mM) produced slight, non-significant changes in the magnitude of the CAP evoked by low intensity sound (38 dB SPL; Figs. 9 and 10). PPADS induced a significant suppression of CAP evoked by high intensity sound (92 dB SPL; Figs. 9 and 10) starting at 1.0 mM with no recovery following two washes with AP (Figs. 9 and 10; n = 5 animals). In contrast to CAP magnitude, N1 latency was significantly prolonged at all intensities (Figs. 9 and 10) by 1 mM PPADS and the effect was not reversed by two washes with AP. The most sensitive potential was high intensity sound evoked SP which was suppressed significantly by 0.330 and 1.0 mM PPADS in a reversible manner (Fig. 9 and 10). Surprisingly, low intensity sound evoked SP was less sensitive being reversibly suppressed only by 1 mM PPADS (Figs. 9 and 10). CM was not affected by the PPADS (Figs. 9 and 10).

3.3. Effects of PPADS on DPOAEs.

The typical effect of continuous low level primary stimulation on quadratic (f_2- f_1) DPOAE amplitude is shown in Fig. 11 following the second artificial perilymph perfusion (AP2). Six points (A - F) were identified on each response amplitude function (Fig. 11). These values were used to characterize the response as previously described (Kujawa et al., 1996). They include an initial value (A), an "on-effect" (A - B), a "slow-decline" (B - C), an "off-effect" (C - D) and a "second on-effect" (D - E). Because of the difficulty in defining points B and E, it was

omitted in the statistics. We have published the lack of effect of multiple perfusions of this artificial perilymph on the quadratic DPOAE previously (Kujawa et al., 1996), therefore no multiple artificial perilymph perfusions were carried out in the present study to confirm that the vehicle itself induced no change.

PPADS perfused (15 min) through the cochlea in a cumulative dose manner significantly (330 and 1 mM) reduced the absolute amplitude of the quadratic distortion product measured after the 15 min of silence at point A, the "initial value" (Figs. 11 and 12; n = 6 animals). Also, PPADS had such complex effects on the 'on effect' that it was not treated statistically (point B). In addition, PPADS attenuated (0.330 mM) or reversed (0.33 to 1 mM) the 'slow-decline'(points A - C) in the quadratic DPOAE induced by continuous, moderate primary stimulation (Figs. 11 and 12; n = 6 animals). The reversal following 1 mM PPADS is seen in Fig. 12 as a statistically significant suppression of point A while point C is not significantly different from control (AP2).

The amplitude growth functions for the quadratic DPOAE (f_2-f_1 = 1.25 kHz) obtained immediately after the PPADS perfusions and the above measurements (i.e., Fig. 11) were unchanged compared to those obtained after the AP2 values except for two values (i.e., an increase in the 30 dB SPL at 1 mM; a decrease in the 70 dB SPL value at 0.33 mM; Figs. 13 and 14). The general lack of effect of 1 mM PPADS most probably reflects the PPADS induced attenuation and reversal of the 'slow-decline' in the quadratic DPOAE during continuous exposure to the primaries. In addition, there were statistically significant increases at only three points in the amplitude growth functions of the cubic DPOAE ($2f_1$-f_2 = 5 kHz; i.e., 35 and 55 dB SPL at 0.33 mM; 35, 55 and 60 dB SPL at 1.0 mM: Figs. 13 and 14). In a few individual animals, the growth functions of both the quadratic and cubic DPOAE were shifted to the left (Bobbin et al., 1997a), but the large variance in the occurrence of this shift resulted in few statistically significant changes in the mean values.

4. Discussion

Results demonstrate that PPADS, a drug previously shown to block ATP at P2X1, P2X2, P2X3, and P2X5 ionotropic receptors in other systems (Buell et al., 1996; Collo et al., 1996; Ziganshin et al., 1995), reduces ATP gated currents in acutely isolated OHCs, Deiters' cells, Hensen's cells and pillar cells. To the best of our knowledge this is the first report demonstrating the presence of ionotropic ATP receptors on pillar cells and the block of the ionotropic effects of ATP by PPADS in pillar cells, Deiters' cells, OHCs and Hensen's cells. In addition, this is the first report demonstrating that the peak current induced by ATP is increased when coapplied with PPADS in supporting cells. However, the ATP evoked current in response to the subsequent application of ATP alone was greatly reduced indicating that the onset of the PPADS block is slow, consistent with the results reported by others (Buell et al., 1996). The ATP induced response was almost completely blocked by a pretreatment with PPADS alone suggesting that PPADS is not an open channel blocker. The ionotropic effect of ATP described here confirms previous work of others on OHCs (Ashmore and Ohmori, 1990; Chen et al., 1995a,b, 1997; Dulon et al., 1993; Dulon, 1995; Housley et al., 1992; Kakehata et al., 1993; Kujawa et al., 1994b; Nakagawa et. al., 1990; Nilles et al., 1994) and Deiters' cells (Chen et al., 1997; Dulon, 1995; Skellett et al., 1997) and Hensen's cells (Sugasawa et al., 1996b).

In the cochlea, the P2X2 like nucleotide (mRNA) sequence has been localized to rat Deiters' cells (Glowatzki et al., 1995), rat organ of Corti (Housley et al., 1995; Brandle et al., 1997) and guinea pig organ of Corti (Parker et al., 1997). To date, P2Y ATP receptors (metabotropic or G protein coupled) have been only indirectly suggested to be present in the cells of the organ of Corti (Deiters' cells, Dulon, 1995; Hensen's cells, Sugasawa et al., 1996b), while stronger evidence for their presence in the stria vascularis has been presented (Liu et al., 1995; Suzuki et al., 1995; White et al., 1995). Both the prolonged onset and wash out that we observed

with PPADS in vitro on OHCs, Deiters' cells, Hensen's cells and pillar cells are characteristic of some types of the P2X receptor proteins, such as the P2X2 (Buell et al., 1996; Collo et al., 1996). The slow kinetics of these P2X receptors have been attributed to the presence of lysine at position 246 in the receptor protein (Buell et al., 1996; Collo et al., 1996). Parker et al. (1997) reported an arginine, which should behave similarly to lysine, in position 246 in the P2X2 sequence they found in guinea pig organ of Corti. The relatively faster wash out of PPADS from Hensen's cells may indicate that a P2X receptor protein significantly different from the one present in OHCs, Deiters's cells and pillar cells is present in Hensen's cells. Whether this is the case will be determined by future studies of the distribution of various P2X receptors in the cells of the organ of Corti.

PPADS, had no effect on voltage gated currents recorded from OHCs, Deiters' cells, Hensen's cells or pillar cells. Suramin also had no effect on these currents in OHCs or Deiters' cells (Skellett et al., 1997). Cibacron blue, an additional ATP antagonist, has been demonstrated to have large effects on voltage activated potassium currents in both OHCs and Deiters' cells (Skellett et al., 1997). Cubic DPOAEs are sensitive to changes in ion concentrations in perilymph and to drugs that affect ion channels (e.g., Kujawa et al., 1996). Cibacron (Kujawa et al., 1994b) but not PPADS or suramin reduced the magnitude of the cubic DPOAE. The minimal effect of PPADS and lack of effect of suramin on the cubic DPOAE may be additional evidence that in contrast to cibacron, PPADS and suramin have no effects on voltage gated ion channels in cells in the organ of Corti. Overall it appears that both suramin and PPADS are relatively more selective than cibacron blue in blocking the ionotropic P2X type of ATP receptor protein (Skellett et al., 1997).

It appears that the efferent transmitters, acetylcholine and GABA, are not involved in generating the time varying amplitude changes of the quadratic DPOAE that occur during

continuous stimulation with moderate level primaries (Kirk and Johnstone, 1993; Lowe and Robertson, 1995; Kujawa et al. 1995, 1996). In a previous study (Skellett et al., 1997), suramin perfused through the perilymph compartment attenuated or reversed the slow decline in the time varying amplitude changes of the quadratic DPOAE. Therefore, it was tentatively suggested that possibly extracellular ATP acting as a neuromodulator was involved in the amplitude changes of the quadratic DPOAE (Skellett et al., 1997). Due to the reversal of the slow decline by suramin, the magnitude of the subsequently recorded intensity functions of the quadratic DPOAEs were enhanced. The cubic DPOAE was not affected by suramin (Skellett et al., 1997). In the present study, PPADS reduced the initial value of the quadratic DPOAE (point A in Fig. 11) which was not observed with suramin (Skellett et al., 1997). Like suramin, PPADS attenuated the slow decline at 0.33 mM and reversed the decline into a slow rise (1 mM; Fig.11). However, the slow changes induced by PPADS were superimposed on the drop in the initial value (point A in Fig. 11), so the final values (point F in Fig. 11 for 1 mM PPADS) did not exceed the control final values (point F in Fig. 11 for AP2). We guess that this is the reason for the lack of a general overall enhancement of the subsequently recorded intensity functions of the quadratic DPOAE following PPADS. The differences between the effects of the two drugs may simply reflect the greater potency of PPADS in blocking ATP (Collo et al., 1996; Skellett et al., 1997). The fact that PPADS affects the time varying amplitude changes of the quadratic DPOAE that occur during continuous stimulation with moderate level primaries in a manner that is similar to suramin may be taken as additional evidence that ATP acting as a neuromodulator plays a role in generating the amplitude changes.

In the present study, the cochlear potentials were affected by PPADS as they were affected by suramin in the study by Kujawa et al. (1994b). Both drugs had no effect on CM and suppressed high intensity SP. PPADS suppressed high intensity SP to a greater degree than

suramin and PPADS had a slightly significant effect on low intensity SP while suramin did not. Therefore, it appears that, in general, PPADS had a similar but greater effect on SP than suramin. This would be predicted from the fact that PPADS is about twice as potent an ATP antagonist as suramin at the P2X2 type of ATP receptors studied by Collo et al. (1996). PPADS was definitely much more effective than suramin in blocking ATP in OHCs and Deiters' cells in our hands (Skellett et al., 1997). While the ATP antagonists, suramin and PPADS, decrease the value of high intensity sound evoked SP, the ATP agonists, ATP, ATPγS and others, increase it, possibly indicating that both sets of drugs are acting at the same site (Kujawa et al., 1994a). Since the effect of PPADS on SP was readily reversed with washing, then it may be related to the time varying amplitude changes in the quadratic DPOAE which were also readily reversed.

In contrast to the greater potency of PPADS compared to suramin on SP and DPOAEs, suramin was more potent in suppressing CAP and prolonging N1 latency than PPADS. Suramin induced a significant shift in CAP magnitude at 0.1 mM and almost totally abolished the CAP at 1 mM (Kujawa et al., 1994b), whereas PPADS had only a slight effect on CAP and that was at 1 mM and at high intensity sound evoked responses. PPADS did have a fairly large effect on N1 latency at 1mM that was not reversed with washing. Likewise most of the increases in the cubic DPOAE amplitudes induced by PPADS were not reversed with washing (Fig. 14). Thus the effect of PPADS on N1 latency and on the cubic DPOAE amplitudes may be due the same cellular mechanisms. The effect of suramin on CAP may be due to a pharmacological effect that is not exhibited by PPADS.

In the previous study of suramin (Skellett et al., 1997) and in this study of PPADS, cochlear mechanics were monitored by measuring DPOAEs. DPOAEs are generated by a combination of both passive cochlear mechanics and active cochlear mechanics (i.e., the cochlear amplifier). The latter is believed to be brought about by a change in length of the OHCs that are

coupled to Deiters' cells (e.g., Brownell, 1996; Bobbin, 1996; Frank and Kossl, 1996). We argued previously that the action of suramin on cochlear mechanics was due to the blockade of endogenous ATP acting on ionotropic receptors on Deiters' cells (Skellett et al., 1997). This hypothesis was chosen because, although almost every cell in the organ of Corti has ionotropic ATP receptors, many authors have presented evidence that the receptors are on the endolymphatic side of OHCs, Hensen's cells and IHCs and therefore not accessible to suramin delivered to the perilymph (e.g., OHCs, Housley et al., 1992; Deiters' cells, Dulon, 1995; Hensen's cells, Sugasawa et al., 1996b; IHCs, Sugasawa et al., 1996a). As shown here even pillar cells have these receptors but the receptor location was not determined. If the receptors on any of the cells are on a portion of the cells bathed by perilymph, it is possible that the in vivo effects of PPADS and suramin on the DPOAEs described here may be due to blockade of ATP activation of those receptors. Therefore, a definitive statement as to the cells responding to PPADS and suramin in vivo cannot be made until the receptors are localized. However, we speculate that given the successful wash out of the effects of PPADSs on the time varying amplitude change in the quadratic DPOAE and SP, it is possible that these effects were due to the block of endogenous ATP where the in vitro effects of PPADS were readily reversed (e.g., Hensen's cells). In contrast, the effect of PPADS on N1 latency, CAP amplitude and cubic DPOAE intensity functions were not readily reversed and may be due to the block of ATP at other cells in the organ of Corti where the drug was not readily reversed in vitro (e.g., OHCs, pillar cells or Deiters' cells).

The source of the ATP acting on the ionotropic receptors on OHCs, pillar cells, Deiters' cells and Hensen's cells is unknown. Recently, Wangemann (1996) and Wangemann and Marcus (1994) demonstrated release of ATP from cells of the isolated organ of Corti though no particular cell was determined as the release site. OHCs are well known to be innervated by both afferent

and efferent nerve fibers. Burgess et al. (1997) demonstrated nerve fiber innervation of the Deiters' cells and Hensen's cells. Whether these nerve fibers are the source(s) of the ATP acting on ATP receptors on these cells in the organ of Corti has not been demonstrated. No innervation of pillar cells has been demonstrated. On the other hand, the source of the ATP may not be neuronal since acute efferent nerve sectioning and tetrodotoxin have no effect on the time varying amplitude change in the quadratic DPOAE (Lowe and Robertson, 1995; Kujawa et al. 1995, 1996).

In summary, in this study, PPADS, exhibited some of the same actions as were found for suramin in a previos study by Skellett et al. (1997), together with some differences. Both drugs blocked the ATP induced inward currents in OHCs, and Deiters' cells without an affect on the voltage activated currents in these cells. In addition, PPADs blocked ATP induced currents in pillar cells and Hensen's cells. In vivo suramin attenuated and reversed the 'slow-decline' in the amplitude of the quadratic DPOAE induced by moderate, continuous primary stimulation without an effect on the 'initial value'. PPADS suppressed the 'initial value' and attenuated or reversed the 'slow-decline'. Both PPADS and suramin reduced the magnitude of the SP. The effects of PPADS on the quadratic DPOAE and SP were readily reversed with washing. In addition, PPADS suppressed high intensity CAP, N1 latency at all intensities and enhanced the cubic DPOAE at a few intensities. These later actions of PPADS were not reversed with washing and therefore may be due to actions of PPADS at sites different from those inducing the reversible responses. In general, we speculate that the ATP antagonists, suramin and PPADS, block endogenous ATP acting on ionotropic ATP receptors on cells in the organ of Corti and so induce the effects observed.

Acknowledgments

Thanks to Latasha Bright, Julie Campbell and Michele Larroque for technical assistance and Emma Bockman, Ph.D. for encouraging RPB to study ATP in the cochlea. This work was supported in part by research grant numbers R01 DC 00722 and PO1 DC00379 from the National Institute on Deafness and Other Communication Disorders, National Institutes of Health, and DAMD 17-93-V-3013, Kam's fund for Hearing Research, and the Louisiana Lions Eye Foundation.

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Fig. legends:

Fig. 1. Effects of PPADS on ATP gated current in OHCs. **a**. ATP (10 μM) induced inward current recorded from an OHC when applied by the U-tube for 10 s as indicated by the horizontal bar. **b**. Co-application of ATP (10 μM) and PPADS (100 μM) in the same cell as in **a** reduced the ATP induced current slightly. **c**. ATP (10 μM) induced current response recorded following 1 min of washing out the co-applied drugs in **b** is reduced compared to the response to ATP in **a**. **d**. ATP induced current response following 10 min of the wash demonstrates no recovery of the response to ATP. Current traces from **a** to **d** are from the same cell and Vh was -60 mV. **e**. ATP (10 μM) induced inward current recorded from another OHC. **f**. Application of PPADS (100 μM) alone by the U-tube for 10 s. **g**. ATP induced current response is blocked when applied following 1 min of washing out the PPADS. **h**. ATP induced current following 10 min of washing demonstrates no recovery. Current traces from **e** to **h** are from the same cell and Vh was -60 mV. The dashed line is inserted as a visual aid only.

Fig. 2. Lack of effect of PPADS on voltage-gated currents in OHCs. Current was elicited by 100 ms steps from -120 to 50 mV from a holding potential of -60 mV. **a** and **b** show current records in the absence and presence of PPADS (100 μM). **c**. No difference is seen in the current-voltage (I-V) relationships constructed from **a** and **b**. Current amplitude was measured 50 ms after the onset of the pulses.

Fig. 3. Effects of PPADS on ATP-gated currents in Deiters' cells. **a**. ATP (5 μM) induced inward current recorded from a Deiters' cell. **b**. Co-application of ATP (5 μM) and PPADS (100 μM) enhanced the peak current induced by ATP but reduced the duration of the current. **c**. ATP (5 μM) induced current response recorded following 1 min of washing out the co-applied drugs in **b** is almost completely blocked compared to the response to ATP in **a**. **d**. ATP induced current following 10 min of the wash demonstrates some recovery of the response to ATP. Current traces from **a** to **d** are from the same cell and Vh was -80 mV. **e**. ATP (5 μM) induced inward current recorded from another Deiters' cell. **f**. Application of PPADS (100 μM) alone by the U-tube for 10 s. **g**. ATP induced current response is blocked when applied following 1 min of washing out the PPADS. **h**. ATP induced current following 20 min of washing demonstrates some recovery. Current traces from **e** to **h** are from the same cell and Vh was -80 mV. The dashed line is inserted as a visual aid only.

Fig. 4. Lack of effect of PPADS (100 μM) on voltage-gated currents in Deiters' cells. **a**. Current traces recorded from a Deiters' cell in the absence and presence of PPADS (100 μM). Current was evoked by 100 ms steps from -80 to 60 mVand repolarized to -40 mV from a holding potential of -70 mV. **b**. Current-voltage (I-V) curves were constructed from records in **a** measured at 2 ms before the termination of varying votage steps. **c**. Superimposed tail current traces recorded in the absence and presence of PPADS (100 μM). **d**. Tail activation curves constructed from current records in **c**. Tail current amplitude was measured 3.5 ms after the termination of the varying voltage steps. The tail activation curves were best fitted to a double Boltzmann function. The parameters for the fits: V_{h1} =-6 mV, K_1 = 8 mV, V_{h2} = 35 mV, and K_2 = 12 mV for the control; and V_{h1} =-6 mV, K_1 =7 mV, V_{h2} = 36 mV, and K_2 = 12 mV for the PPADS.

Fig. 5. Effects of PPADS on ATP gated current in Hensen's cells. **a**. ATP (5 μM) induced a biphasic inward current in a Deiters' cell. **b**. Co-application of ATP (5 μM) with PPADS (100 μM) enhanced the peak current induced by ATP but abolished the biphasic nature of the response compared to the response to control ATP shown in **a** . **c**. ATP (5 μM) induced current response recorded following 1 min of washing out the co-applied drugs in **b** is reduced compared to the response to control ATP in **a**. **d**. ATP induced current response following 12 min of the wash demonstrates complete recovery. **e**. ATP (5 μM) induced inward current response following 20 min of wash. **f**. Application of PPADS (100 μM) alone by the U-tube for 10 s. **g**. ATP induced current response is blocked when applied following after 1 min of washing out the PPADS. **h**. ATP induced current response following 20 min of washing demonstrates a large amount of recovery. Membrane potential was held at -80 mV. Current traces from **a** to **h** are from the same cell. The dashed line is inserted as a visual aid only.

Fig. 6. Lack of effect of PPADS on the voltage-gated currents in Hensen's cells. **a** and **b**. Current traces recorded from a Hensen's cell in the absence (**a**) and presence (**b**) of PPADS (100 μM). Current was evoked by 100 ms steps from -80 to 60 mV and repolarized to -40 mV from a holding potential of -70 mV. **c**. I-V curves were constructed from **a** and **b** measured at 2 ms before the termination of the varying voltage steps.

Fig. 7. Effects of PPADS on ATP-gated currents in pillar cells. **a**. ATP (5 μM) induced inward current recorded from a pillar cell. **b**. Co-application of ATP (5 μM) with PPADS (100 μM) enhanced the peak current induced by ATP but reduces the duration of the response compared to control ATP shown in **a**. **c**. ATP (5 μM) induced current response recorded following 1 min of washing out the co-applied drugs in **b** is almost completely blocked. **d**. ATP induced current response following 20 min of wash demonstrates some recovery. Current traces from **a** to **d** are from the same cell and Vh was -80 mV. **e**. ATP (5 μM) induced inward current recorded from another pillar cell. **f**. Application of PPADS (100 μM) alone by the U-tube for 10 s. **g**. ATP induced current response is blocked when applied following 1 min of washing out the PPADS. **h**. ATP induced current following 30 min of washing demonstrates some recovery. Current traces from **e** to **h** are from the same cell and Vh was -80 mV. The dashed line is inserted as a visual aid only.

Fig. 8. Lack of effect of PPADS (100 μM) on voltage-gated currents in pillar cells. **a** and **b**. Current traces recorded from a pillar cell in the absence (**a**) and presence (**b**) of PPADS. Current was evoked by 100 ms steps from -100 to 60 mVand repolarized to -40 mV from a holding potential of -70 mV. **c**. I-V curves were constructed from **a** and **b** measured at 2 ms before the termination of the varying voltage steps.

Fig. 9. Effect of PPADS on CAP, N_1 latency, SP and CM as a function of stimulus intensity. Shown are functions recorded after pre-drug artificial perilymph perfusion #2 (AP2; 15 min), after perfusion with increasing concentrations (0.033 - 1.0 mM; 15 min each) of PPADS, and after a second post-drug wash with artificial perilymph (W2; 15 min). Functions recorded after 0.033 and 0.1 mM PPADS and wash 1 are not shown for clarity. Data are represented as means ± S.E. across 5 animals.

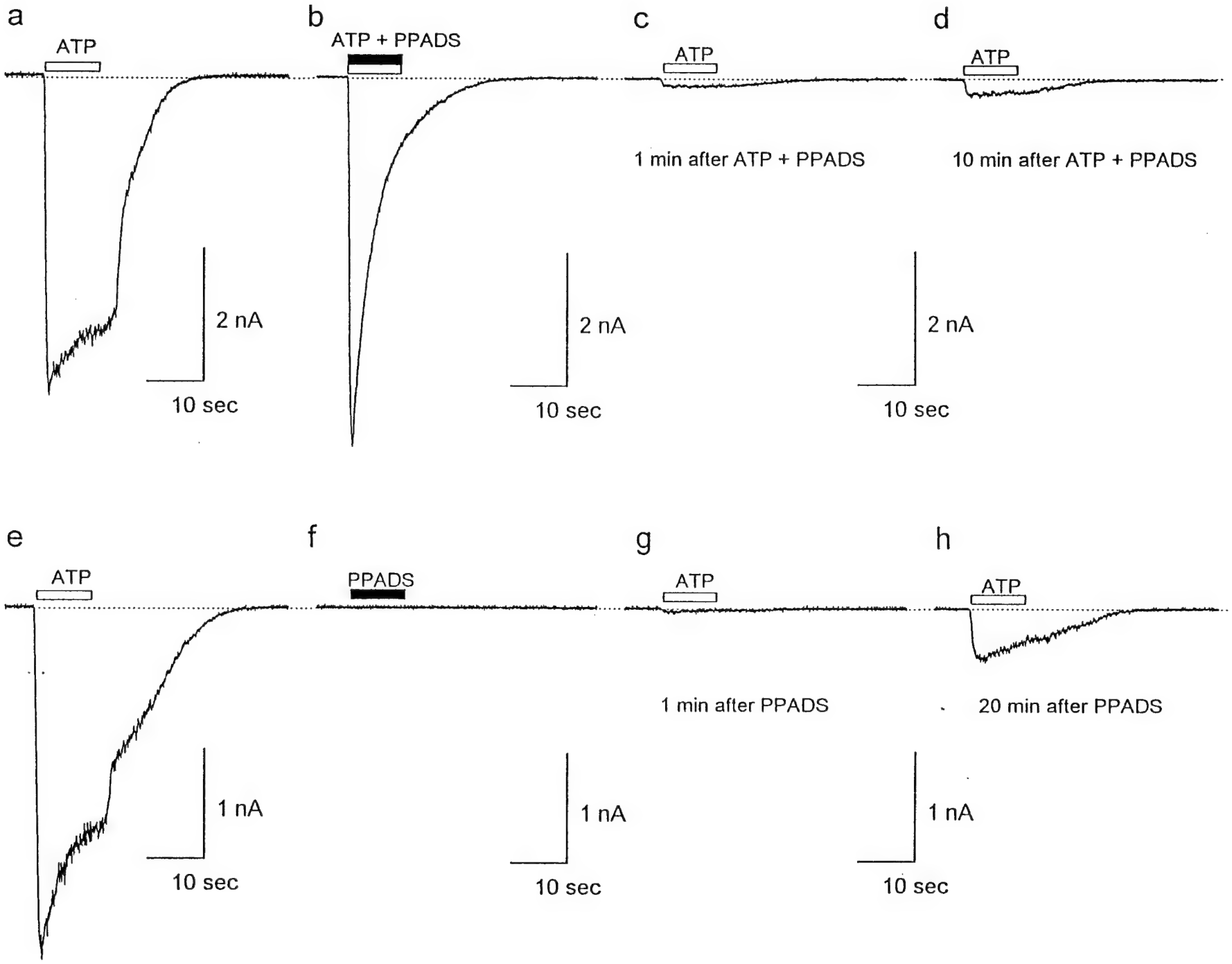
Fig. 10. Intensity dependence of PPADS effects on CAP, N1 latency, SP and CM. Shown are the data representing responses evoked by tone bursts at 38 dB SPL and 92 dB SPL from Fig. 9 after the control perfusion (AP2, 15 min), after perfusion of increasing concentrations of PPADS (0.033 - 1.0 mM, 15 min each) and after artificial perilymph wash perfusions (W1, 15 min; W2, 15 min). Data are displayed as means ± S.E. across n = 5 animals. Values significantly different from AP2 values are designated * $P < 0.05$; ** $P < 0.01$. Responses that fell into the noise floor were assigned the numerical value of the noise floor.

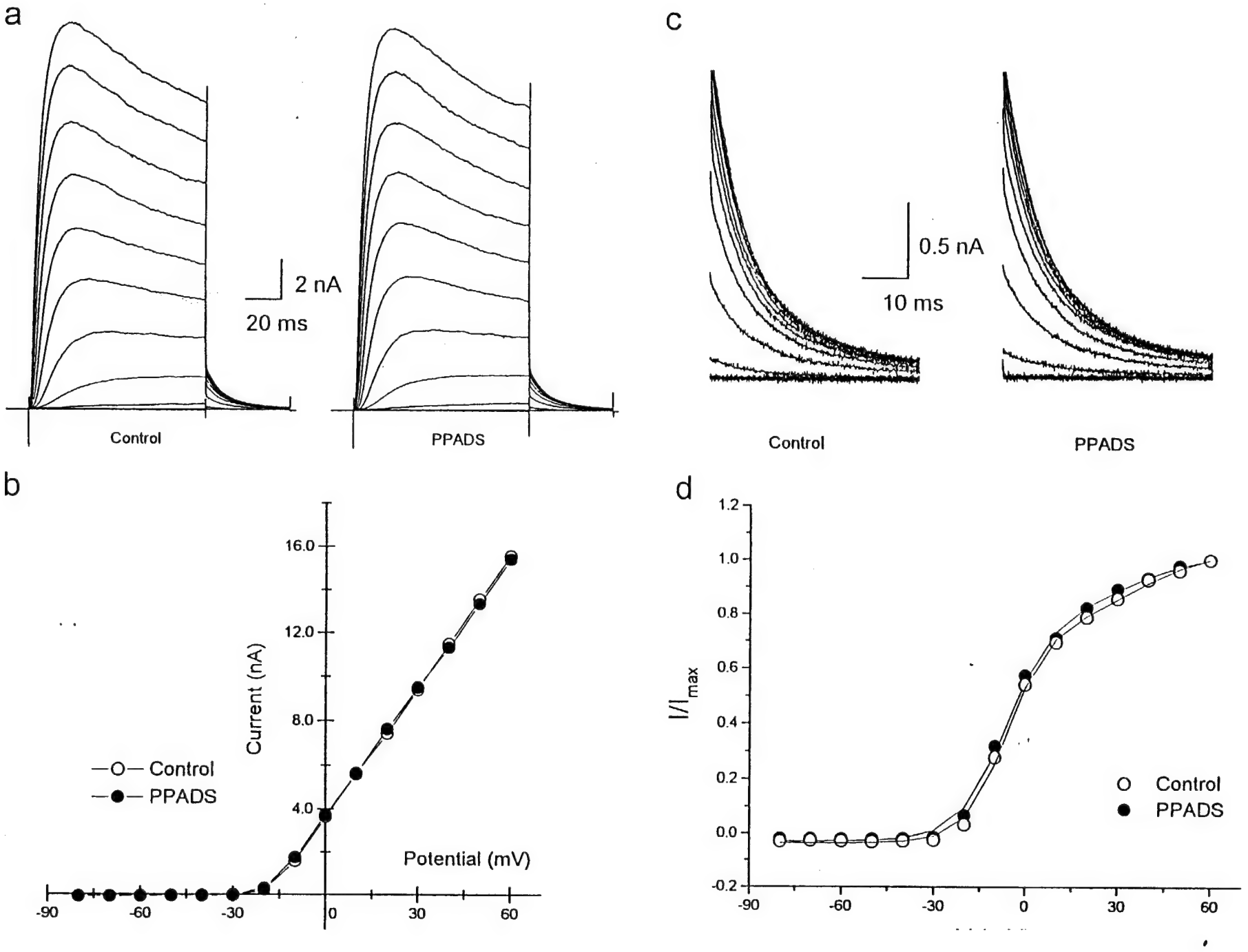
Fig. 11. Effect of PPADS on quadratic f_2 -f_1 DPOAE (f_2 - f_1 = 1.25 kHz) amplitude alterations during continuous primary stimulation (f_1 =6.25 kHz, f_2 = 7.5 kHz, L1 = L2 = 60 dB SPL). Response amplitude as recorded following the second control perfusion (AP2), following increasing concentrations of PPADS (0.033 to 1.0 mM), and a post drug wash (W1). The AP2 trace is repeated in each frame for reference. Pooled errors are shown in the upper right hand corner of each frame. Each data point represents a 10-spectra average and required 5 s to complete. The break in response amplitude trace (C-D) represents 1 min during which the primaries were turned off. Points A - F were used to calculate magnitudes of component amplitude changes for statistical analysis in Fig. 12. Symbols represent means of n = 6. Noise floor averaged about -12 dB.

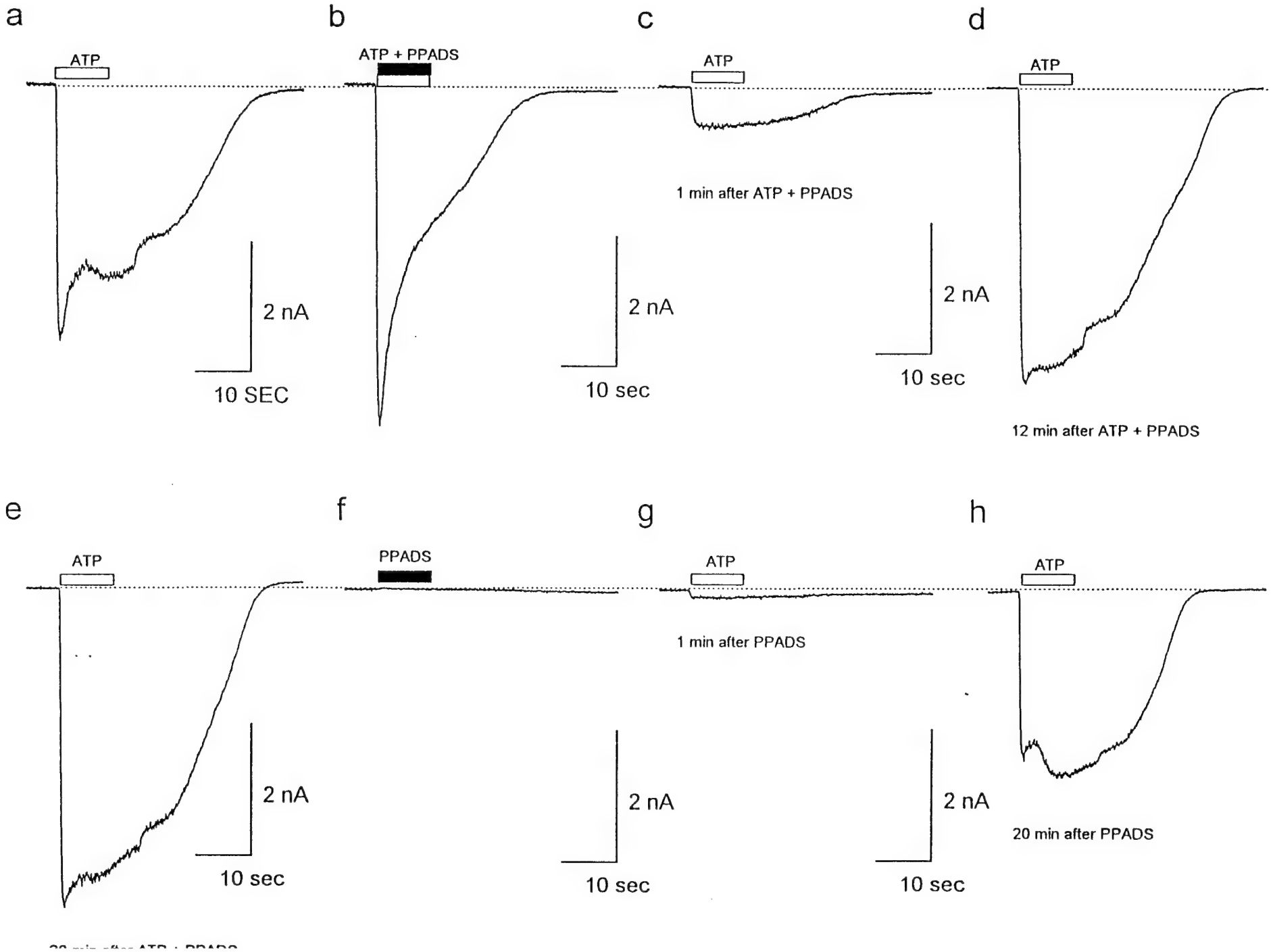
Fig. 12. Effect of PPADS on the various points (A - F; but not B or E) identified in the f_2-f_1 DPOAE amplitude values (defined in Fig. 11) following each perfusion shown in Fig. 11. Significance was tested with ANOVA and subsequent Student-Newman Keuls post hoc multiple range test for all means. Since points B and E were difficult to define for the 1 mM PPADS it was omitted from the calculations and no value is given in the bar graph. Values that are significantly different from their respective AP2 values are designated: * $P<0.05$; ** $P<0.01$. Data are represented as means ± S.E. (n = 6).

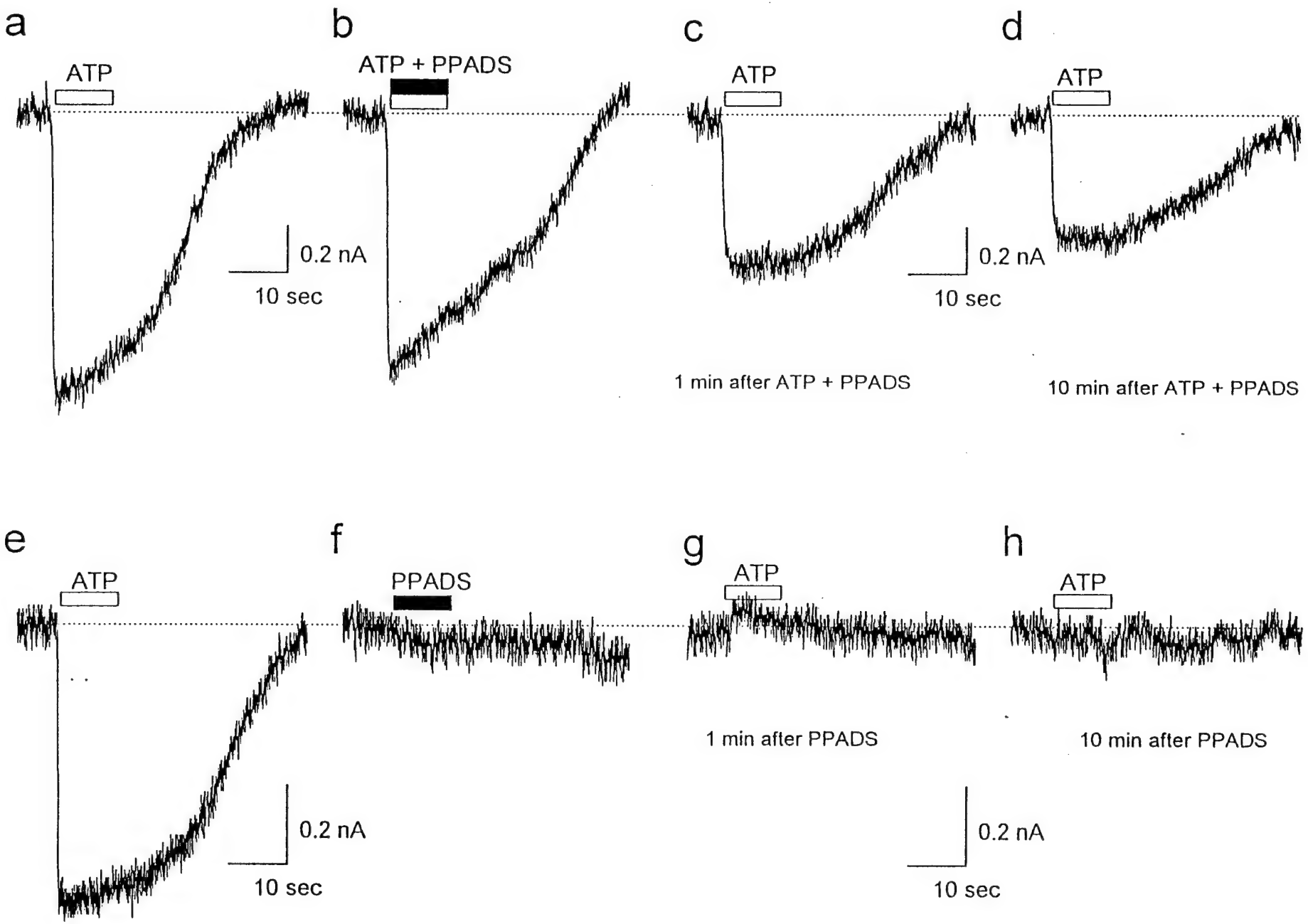
Fig. 13. Effect of PPADS on the amplitude growth functions for quadratic (f_2-f_1) and cubic ($2f_1$ -f_2) DPOAEs. Shown are the values obtained following the second control perfusion (AP2), following increasing concentrations of PPADS (0.033 to 1.0 mM), and a post drug wash (W1) and collection of the data shown in Fig. 11. Functions recorded after 0.033 and 0.1 mM PPADS and wash 1 are not shown for clarity. Data are represented as means ± S.E (n = 6). The dashed line in each panel represents the average value of the noise floor (NF).

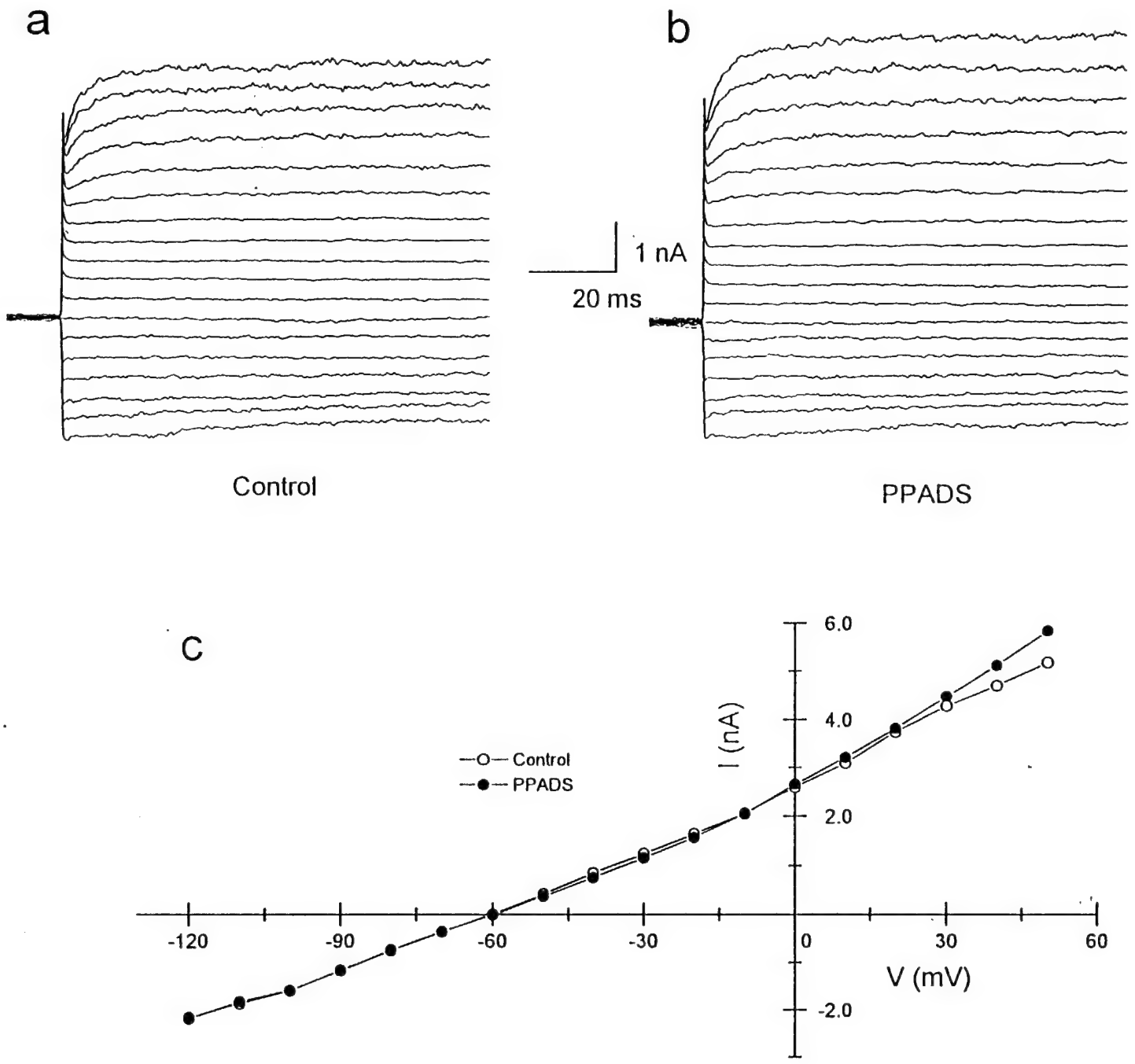
Fig. 14. Intensity dependence of PPADS effects on the quadratic (f2-f1) and cubic 2(f1-f2) DPOAE. Shown are the data representing responses evoked by continuous primaries at 30, 55 and 70 dB SPL for the quadratic and at 35, 55, and 60 dB SPL for the cubic DPOAE from Fig. 13 after the control perfusion (AP2, 15 min), after perfusion of increasing concentrations of PPADS (0.033 - 1.0 mM, 15 min each) and after an artificial perilymph wash perfusion (W1, 15 min). Data are displayed as means ± S.E. (n = 6 animals). Values significantly different from AP2 are designated * $P < 0.05$; ** $P < 0.01$. Responses that fell into the noise floor were assigned the numerical value of the noise floor.

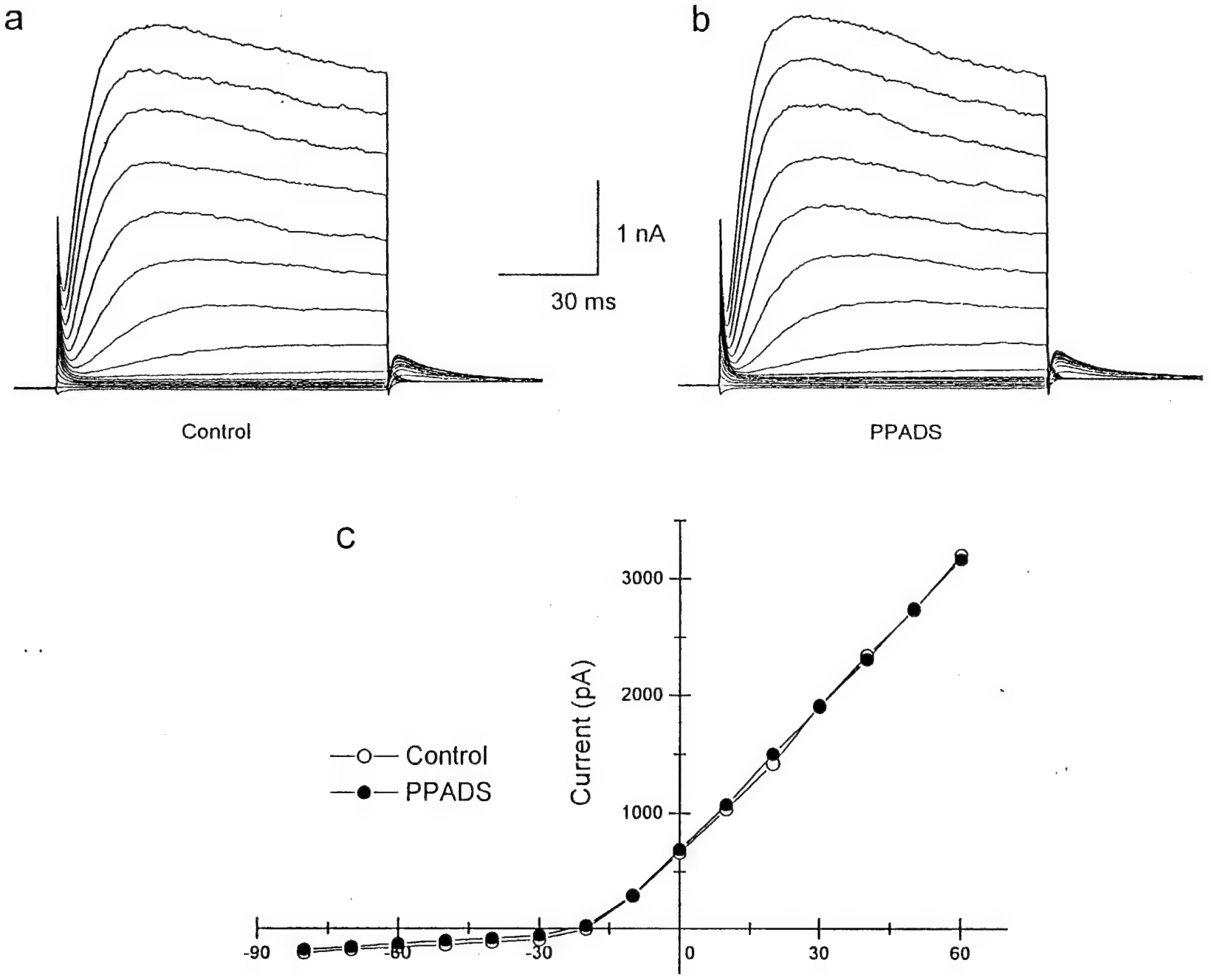

a
ATP
2 nA
10 sec
b
ATP + PPADS
2 nA
10 sec
c
ATP
1 min after ATP + PPADS
2 nA
10 sec
d
ATP
10 min after ATP + PPADS
e
ATP
1 nA
10 sec
f
PPADS
1 nA
10 sec
g
ATP
1 min after PPADS
1 nA
10 sec
h
ATP
20 min after PPADS
1 nA
10 sec

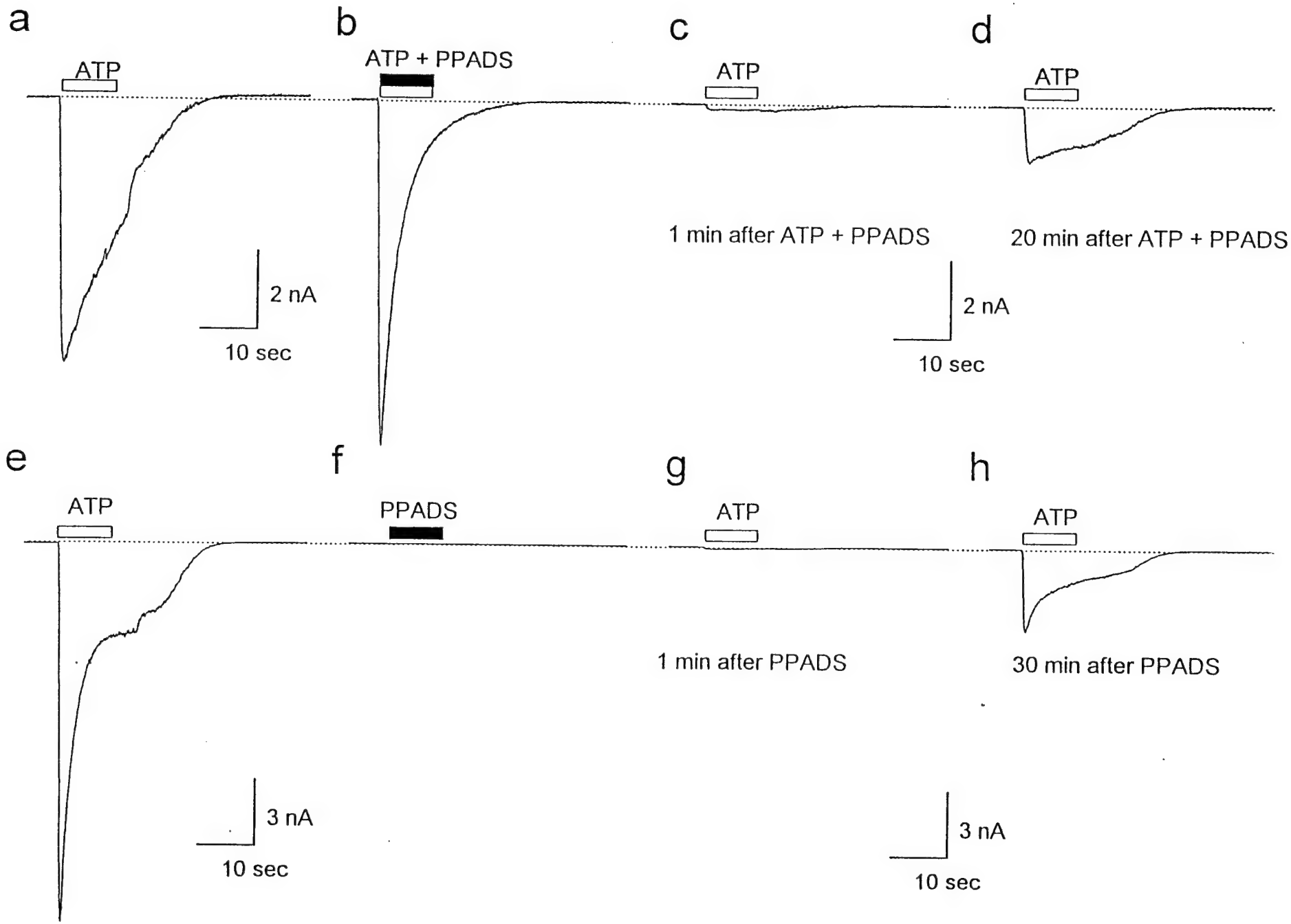

a
2 nA
20 ms
Control
PPADS
c
0.5 nA
10 ms
Control
PPADS
b
16.0
12.0
8.0
4.0
Current (nA)
Control
PPADS
-90
-60
-30
0
30
60
Potential (mV)
d
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
I/I max
Control
PPADS
-90
-60
-30
0
30
60

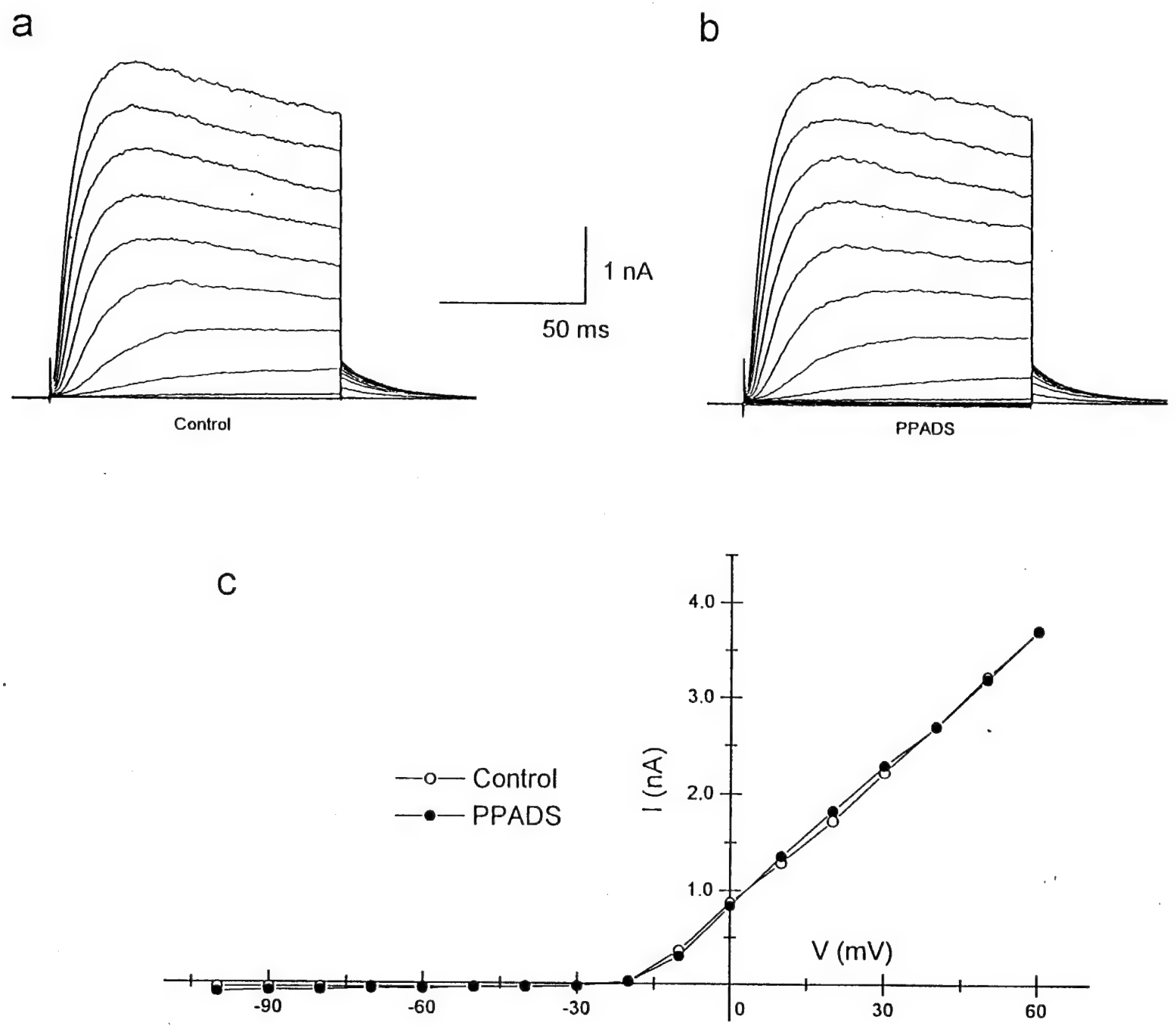

a
ATP
2 nA
10 SEC
b
ATP + PPADS
2 nA
10 sec
c
ATP
1 min after ATP + PPADS
2 nA
10 sec
d
ATP
12 min after ATP + PPADS
e
ATP
2 nA
10 sec
f
PPADS
2 nA
10 sec
g
ATP
1 min after PPADS
2 nA
10 sec
h
ATP
20 min after PPADS

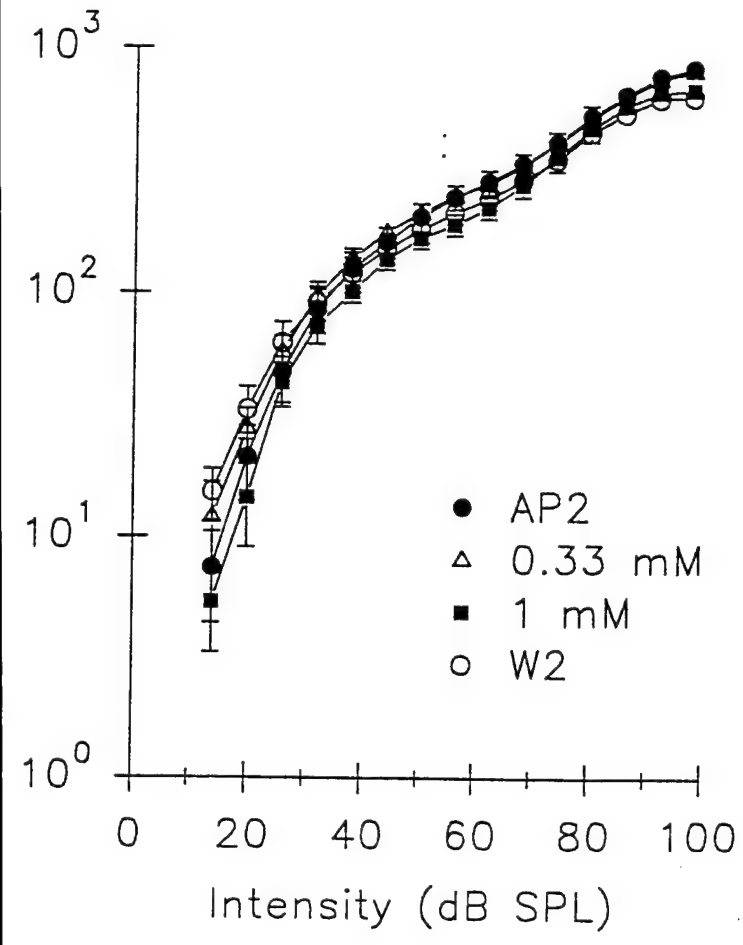

a
ATP
0.2 nA
10 sec
b
ATP + PPADS
c
ATP
1 min after ATP + PPADS
d
ATP
0.2 nA
10 sec
10 min after ATP + PPADS
e
ATP
0.2 nA
10 sec
f
PPADS
g
ATP
1 min after PPADS
h
ATP
10 min after PPADS
0.2 nA
10 sec

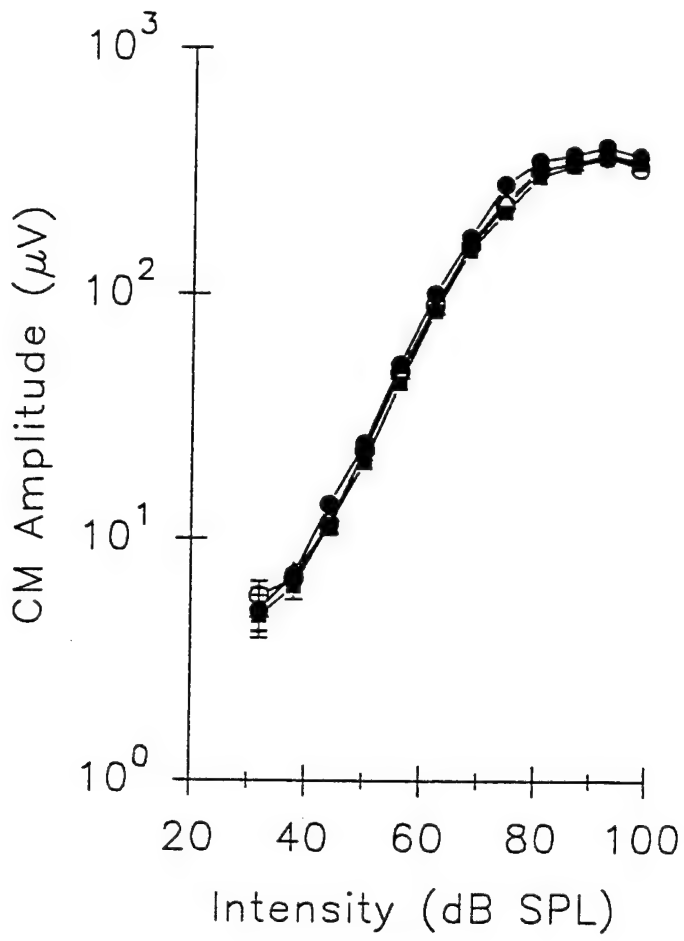

a
b
1 nA
20 ms
Control
PPADS
C
Control
PPADS
I (nA)
V (mV)
6.0
4.0
2.0
-2.0
-120
-90
-60
-30
0
30
60

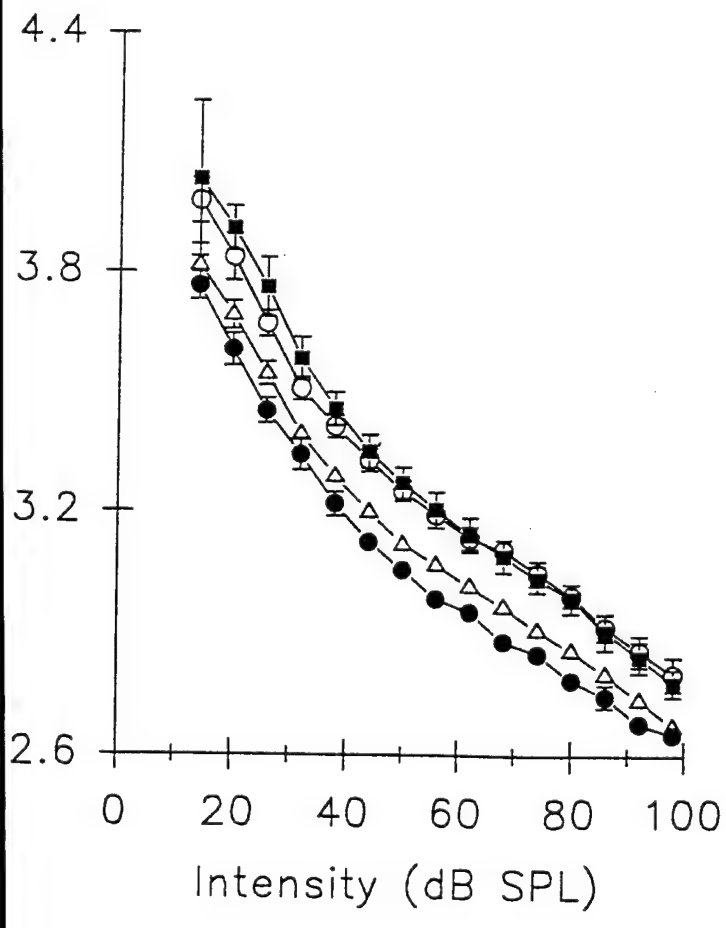

a
b
1 nA
30 ms
Control
PPADS
c
Control
PPADS
Current (pA)
3000
2000
1000
-90
-60
-30
0
30
60

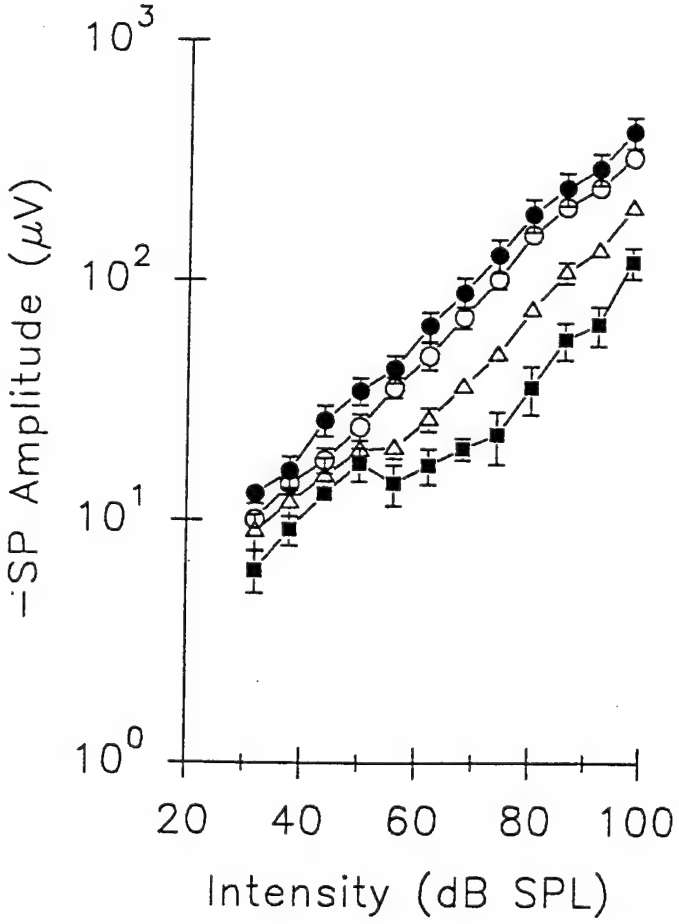

a
ATP
2 nA
10 sec
b
ATP + PPADS
c
ATP
1 min after ATP + PPADS
d
ATP
20 min after ATP + PPADS
2 nA
10 sec
e
ATP
3 nA
10 sec
f
PPADS
g
ATP
1 min after PPADS
h
ATP
30 min after PPADS
3 nA
10 sec

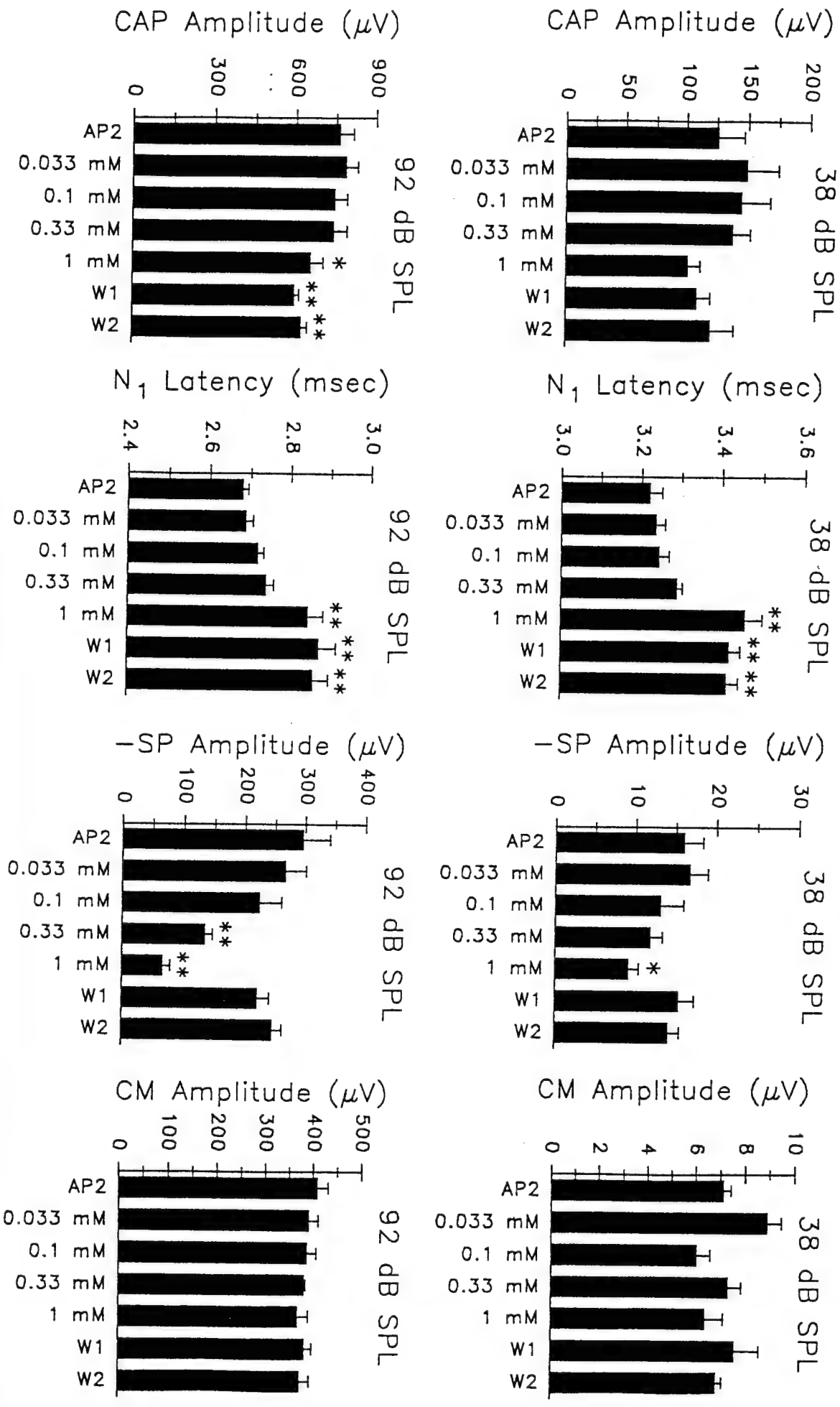

a
b
1 nA
50 ms
Control
PPADS
c
Control
PPADS
I (nA)
V (mV)
4.0
3.0
2.0
1.0
-90
-60
-30
0
30
60

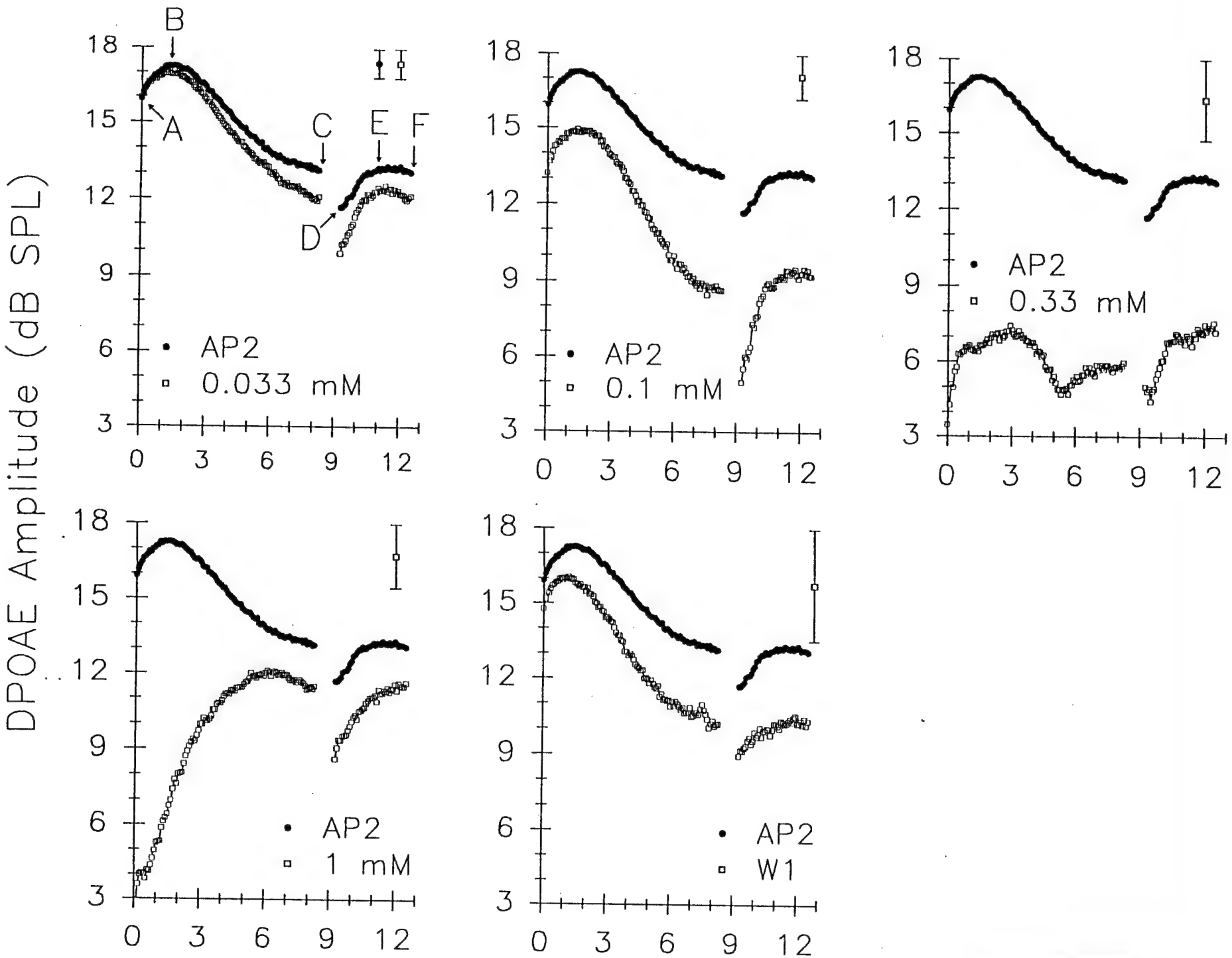

10^3
10^2
10^1
10^0
AP2
0.33 mM
1 mM
W2
0
20
40
60
80
100
Intensity (dB SPL)

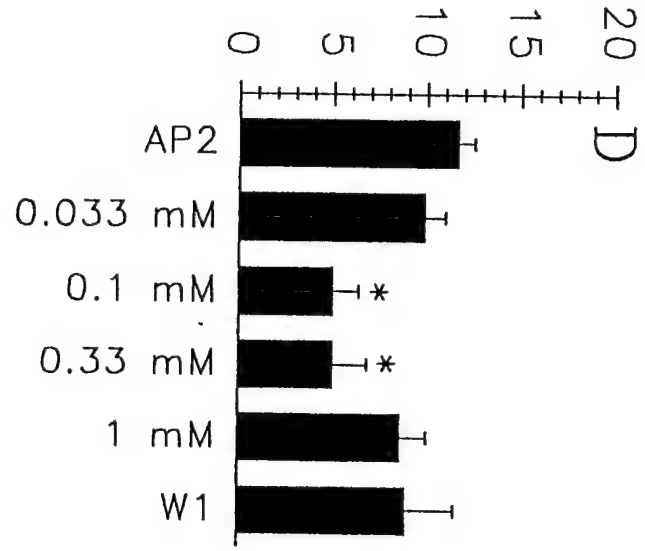

10^3
10^2
10^1
10^0
CM Amplitude (μV)
20
40
60
80
100
Intensity (dB SPL)

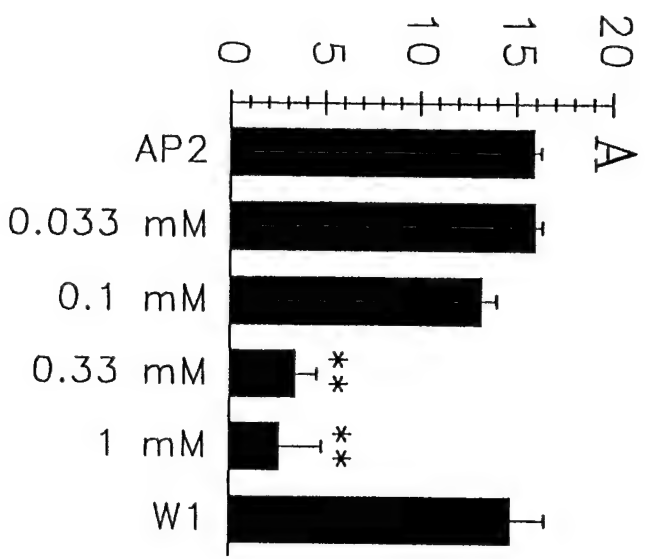

4.4
3.8
3.2
2.6
0
20
40
60
80
100
Intensity (dB SPL)

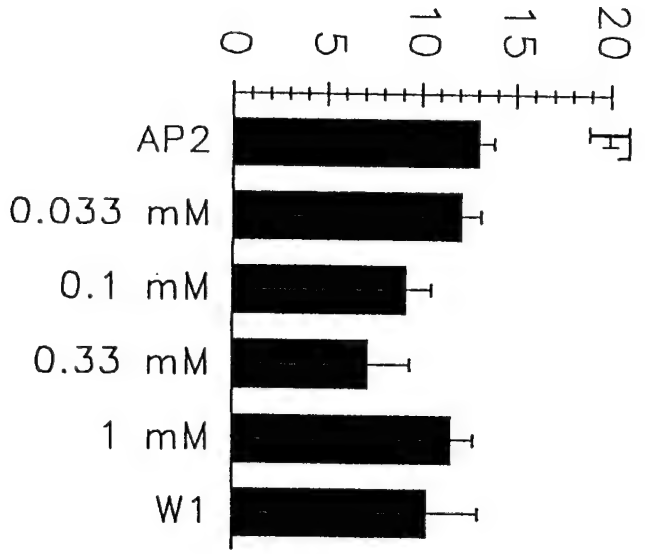

10^3
10^2
10^1
10^0
-SP Amplitude (μV)
20
40
60
80
100
Intensity (dB SPL)

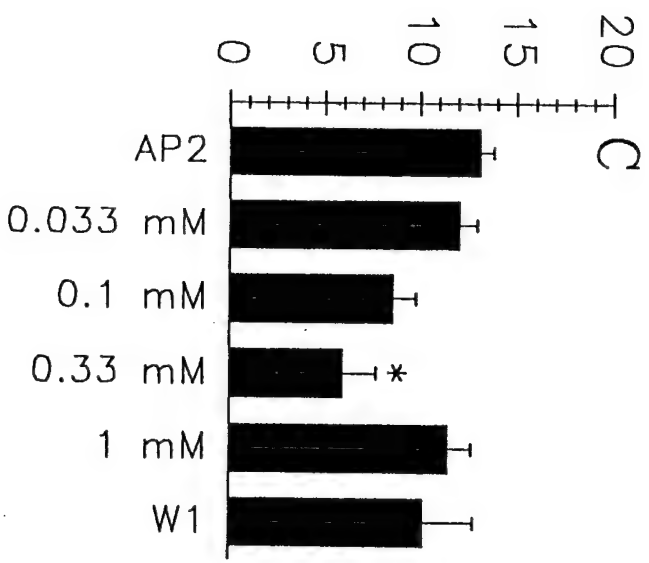

CAP Amplitude (μV)
0
300
600
900
AP2
0.033 mM
0.1 mM
0.33 mM
1 mM
W1
W2
*
**
**
92 dB SPL
CAP Amplitude (μV)
0
50
100
150
200
38 dB SPL
N1 Latency (msec)
2.4
2.6
2.8
3.0
3.2
3.4
3.6
−SP Amplitude (μV)
0
10
20
30
CM Amplitude (μV)
0
2
4
6
8
10

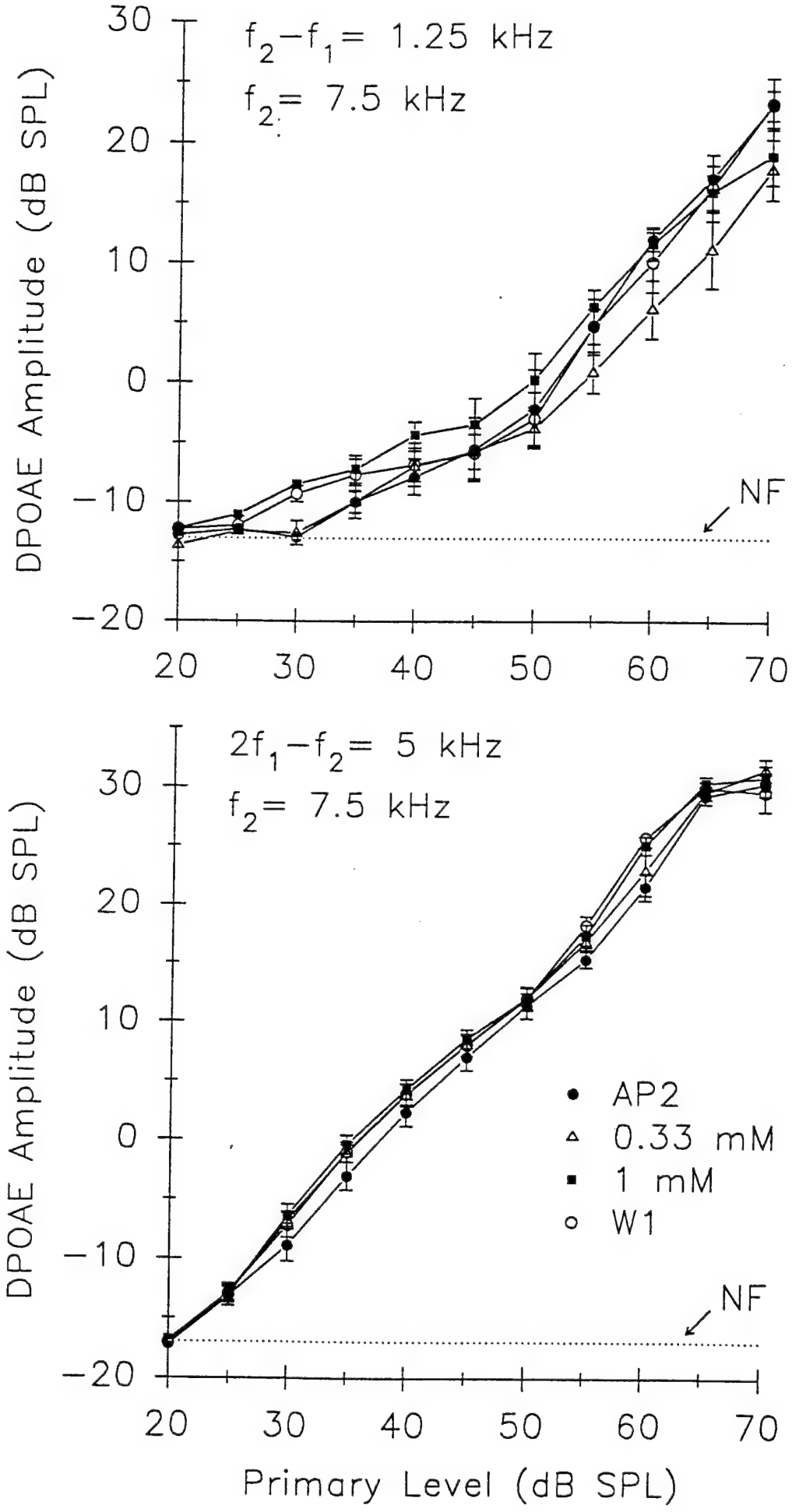

DPOAE Amplitude (dB SPL)
B
A
C
E
F
D
AP2
0.033 mM
AP2
0.1 mM
AP2
0.33 mM
AP2
1 mM
AP2
W1
18
15
12
9
6
3
0
3
6
9
12


0
5
10
15
20
AP2
0.033 mM
0.1 mM
0.33 mM
1 mM
W1
*
*
D

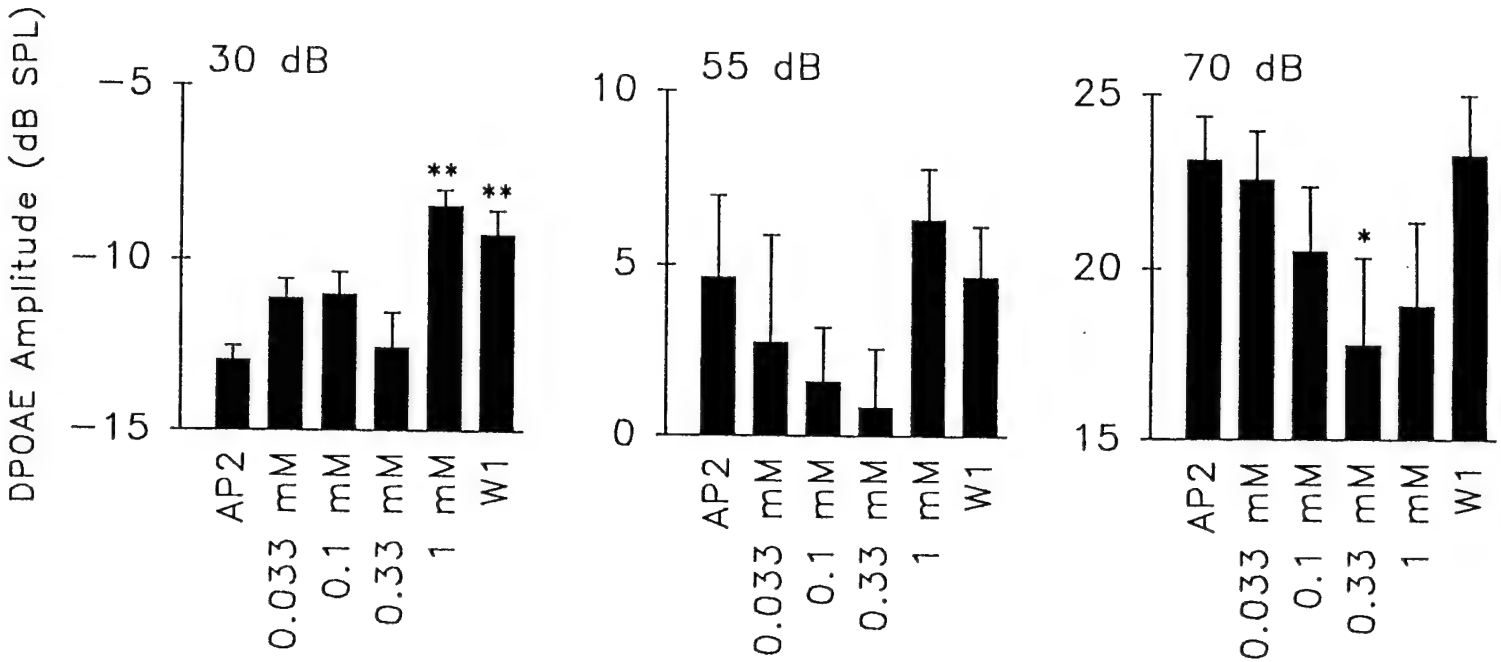

0
5
10
15
20
AP2
0.033 mM
0.1 mM
0.33 mM
1 mM
W1
**
**
A


0
5
10
15
20
AP2
0.033 mM
0.1 mM
0.33 mM
1 mM
W1
H

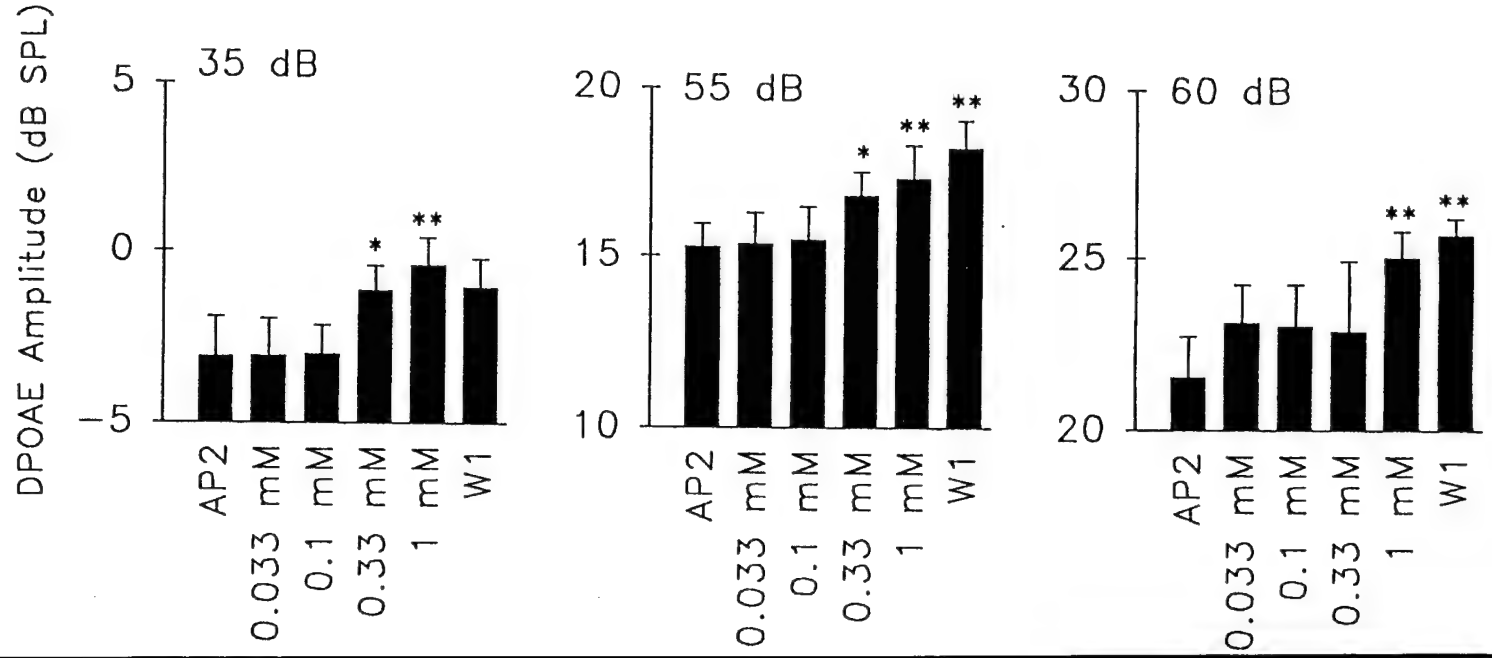

0
5
10
15
20
AP2
0.033 mM
0.1 mM
0.33 mM
1 mM
W1
*
C


f_2-f_1= 1.25 kHz
f_2= 7.5 kHz
DPOAE Amplitude (dB SPL)
30
20
10
0
−10
−20
NF
20
30
40
50
60
70
$2f_1-f_2$= 5 kHz
f_2= 7.5 kHz
AP2
0.33 mM
1 mM
W1
Primary Level (dB SPL)

f_1 = 6,250 Hz
f_2 = 7,500 Hz
$f_2 - f_1$ = 1,250 Hz



f_1 = 6,250 Hz
f_2 = 7,500 Hz
$2f_1 - f_2$ = 5,000 Hz



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IN: Berlin, C.I. (Ed.) Recent Advances in Basic and clinical Hearing Science;

Singlular Press, San Diego, CA

Transmitters in the cochlea: The quadratic distortion product and its time varying response may reflect the function of ATP in the cochlea

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FAX: (504) 568-4460

1. Introduction

Evidence is accumulating to indicate that extracellular adenosine 5' triphosphate (ATP) may function as a neurotransmitter, neuromodulator, mitogen and cytotoxin (Burnstock, 1990; Collo et al., 1996; Wang et al., 1994; Zoeteweij et al., 1996). ATP receptors appear to belong to one super family that is distinct from other receptors. Currently both functional and molecular biology classifications of ATP receptors are being used. The receptors that act by way of a ligand-gated ion channel (ionotropic receptors) are classified as P2X and to date 7 have been sequenced and cloned ($P2X_1$ - $P2X_7$). Those receptors that act by way of a ligand-activated G protein (metabotropic) are classified as P2Y and currently 5 have been identified.

2. The function of ATP in the cochlea.

Bobbin and Thompson (1978) first suggested that ATP and ATP receptors may have a function in the cochlea by demonstrating that extracellular application of ATP to the cells of the cochlea by perfusion of the perilymph compartment affected the function of the cochlea as monitored by a change in the compound action potential of the auditory nerve. Subsequently, ATP-induced alterations in activity of the afferent nerve innervating lateral line hair cells was demonstrated (Bobbin et al., 1979; Mroz and Sewell, 1989). At the single cell level, ATP has been shown to induce ATP receptor

activation (both metabotropic and ionotropic) in several cell types in the cochlea. ATP receptors have been functionally identified in: outer hair cells (OHCs; Ashmore and Ohmori, 1990; Chen et al., 1995a, 1995b; Housley et al., 1992; Kakehata et al., 1993; Kujawa et al., 1994b; Nakagawa et al., 1990; Ashmore and Ohmori, 1990; Ikeda et al., 1991; Shigemoto and Ohmori, 1990); inner hair cells (IHCs; Dulon et al., 1991; Sugasawa et al., 1996); Deiters' cells (Dulon, 1995; Dulon et al., 1993); Hensen's cells (Dulon et al., 1993), and cells of the stria vascularis (Liu et al., 1995; Suzuki et al., 1995; Wangemann, 1995; White et al., 1995; Munoz et al., 1995a; Munoz et al., 1995b).

At the present time, the functions of ATP receptors in the cochlea are unknown (Bobbin, 1996; Eybalin, 1993). Deiters' cells may respond to extracellular ATP with a change in stiffness and through this mechanism ATP may be involved in modulating cochlear mechanics (Dulon, 1995; Bobbin et al., 1997a; Skellett et al., 1997). ATP receptors on the stria may regulate the endocochlear potential (Suzuki et al., 1995; Wangemann, 1995). The ionotropic receptors on OHCs may be involved in transduction, since they appear to be located on the scala media surface of the OHCs (Housley et al., 1992; Mockett et al., 1994 and 1995). ATP may act as a cytotoxin, killing cells when exposed to large amounts of ATP possibly released from killer lymphocytes or during noise exposure (Chu et al., 1997; Bobbin et al., 1997b). ATP may also act as a mitogen, stimulating the proliferation of fibrocytes in the spiral

ligament (Bobbin et al., 1997b).

3. The function of ATP in the organ of Corti: An Hypothesis.

One working hypothesis in our laboratory is that endogenous *ATP modulates cochlear mechanics in the organ of Corti through an action on ionotropic receptors on Deiters' cells*. To prove that any endogenous substance has such a role, a set of criteria has to be met (as discussed in Bobbin et al., 1985). Among these criteria are: (1) the exogenous application of the substance should **mimic** the effects of the endogenously released substance; (2) drugs which block the effects of the endogenous substance should also **block** the effects when the substance is applied exogenously; (3) the substance should be **released** into the extracellular space upon stimulation of the cells of origin; (4) the substance should be **synthesized** in the cells that it is released from; (5) a mechanism for **terminating** the action of the substance should exist; and (6) the **receptor protein and mRNA** for the protein must be present in the cells where the substance is thought to act.

4. Evidence for the Hypothesis.

To monitor cochlear mechanics, we use distortion product otoacoustic emissions (DPOAEs), since they are generated in part

by, and appear to be a reflection of, the active cochlear mechanics in the organ of Corti. Active mechanics involve the outer hair cell-Deiters' cell complex. OHCs shorten and lengthen in response to changes in their resting membrane potential (Bobbin, 1996, Brownell, 1996). Since the OHCs are held by the Deiters' cells at their base and at their apex, then Deiters' cells can alter or modify the movement of the OHCs by a change in their own stiffness. Dulon et al. (1994) demonstrated a change in the stiffness of Deiters' cells in response to a change in intracellular Ca^{2+}. We assume that any change in the mechanics of this cellular complex will be reflected in changes in the DPOAEs (as discussed by Frank and Kossl, 1996). Thus when we refer to changes in cochlear mechanics, we will mean a change in mechanics as monitored through changes in DPOAEs.

4.1 Action of exogenously applied ATP: the mimic criteria.

To test the hypothesis, our first goal was to determine if we could detect an action of exogenously applied ATP on cochlear mechanics as monitored by DPOAEs (Bobbin et al., 1993; Kujawa et al., 1993; Kujawa et al., 1994a). To accomplish this we perfused the perilymph scalae of guinea pigs with artificial perilymph containing ATP and monitored the cubic DPOAE ($2f_1-f_2$) in the ear canal. We placed the drug in the perilymph since it has long been known that drugs readily diffuse from perilymph through the basilar membrane to distribute in the fluid surrounding OHCs and Deiters'

cells. As shown in Figure 1, ATP induced a small suppression of the cubic DPOAEs. It was possible the response to ATP was slight because ATP is rapidly metabolized by ectonucleotidases on the surface of the cells lining the perilymph compartment such as OHCs (Vlajkovic et al.,1996). Therefore, we tested a chemical derivative of ATP, ATP-γ-S, that is resistant to the enzyme and therefore metabolized at a much slower rate than ATP. As shown in Figure 1, the ATP-γ-S was much more potent than ATP and almost abolished the cubic DPOAE at a concentration of 1 mM. Since the drug had little or no effect on the endocochlear potential (EP), it is reasonable to speculate that the drug acted on cells in the organ of Corti, particularly, the OHC - Deiters' cell complex. Both OHCs and Deiters' cells have ATP receptors in their membranes. However, there is strong evidence that the ionotropic ATP receptors on OHCs are located on the endolymph side of the cells (Housley et al., 1992; Mockett et al., 1994, 1995). Dulon (1995) and Dulon et al. (1993) suggest that the ATP receptors on Deiters' cells are located near the region where the cell contacts the base of the OHC, or on the perilymph side of the reticular lamina. If this is the case, then drugs such as ATP-γ-S, when placed in perilymph therefore bathing the base and lateral walls but not the cuticular plate region of the OHCs, can only affect ionotropic ATP receptors on Deiters' cells, but not those on OHCs. These results on DPOAEs were the first evidence that our hypothesis may be correct (criterion #1).

4.2 Detection of an action of endogenously released ATP on mechanics: the blockade criterion

The results with agonists applied into the cochlea present evidence as to the effects of activation of the receptors but do not demonstrate that endogenous ATP is capable of having a similar role in normal or pathological physiology. To accomplish this task physiologists and pharmacologists have traditionally relied on the effects of antagonists. If an antagonist abolishes a physiological response, then this is taken as evidence that the blocked response was mediated by the endogenous substance (criterion #2).

4.2.1 Effect of cibacron and suramin on cubic DPOAEs.

Figure 2, taken from Kujawa et al.(1994b), shows that the ATP antagonist, cibacron blue (cibacron), when perfused through the perilymph compartment of guinea pig cochlea in a manner similar to the ATP perfusion, almost totally abolished the cubic DPOAE. This is evidence that cibacron blocked the actions of endogenous ATP at receptors on cells in the cochlea, that in turn prevented the production of normal DPOAEs. Parenthetically, it is not unusual for both an agonist such as ATP and an antagonist such as cibacron to exhibit similar effects when one is monitoring a remote response like the DPOAEs. For example, both ACh, an agonist, and curare, an antagonist, block the response of the efferents (Bobbin and

Konishi, 1971a, 1971b, 1974). The results with cibacron blocking the generation of the cubic DPOAEs allow us to draw the tentative conclusion that endogenous release of ATP onto ATP receptors, possibly at the OHC-Deiters' cell complex, is necessary for the generation of the DPOAEs.

In the Kujawa et al.(1994b) study, suramin, an additional ATP antagonist, had no effect on the cubic DPOAE, even though it did abolish the compound action potential of the auditory nerve. This effect of suramin on the action potential may have been due to an action on glutamate, the afferent transmitter released from the inner hair cells (Aubert et al., 1995; Bobbin et al., 1985; Bledsoe et al., 1988). So the question remained as to whether suramin was indeed an ATP antagonist at the ATP receptors on OHCs and Deiters' cells.

4.2.2. Action of ATP antagonists (cibacron, suramin and PPADS) at the single cell level.

In order to examine whether cibacron or suramin block the actions of ATP on OHCs and Deiters' cells, these cells were isolated from guinea pig cochlea and whole cell voltage clamp studies of the drugs were carried out. Under these conditions, the cells were studied directly and drug-induced changes in other properties of the cells monitored.

As shown in Figure 3A, application of ATP to a Deiters' cell induced an inward current due to the ATP-induced activation of an ionotropic ATP receptor (Dulon, 1995; Chen et al., 1997). When suramin was applied together with ATP, the response to ATP was reduced (Figure 3A). This indicates that suramin is an antagonist of ATP at ATP receptors on these cells. Figure 3B and 3C illustrate the lack of effect of suramin on the currents induced by stepping the voltage of the cell to different values. Suramin induced no change in these voltage-induced currents indicating that suramin is specific for the ATP receptor protein and has no effect on the voltage activated ion channel proteins.

Results with cibacron were different from suramin. As shown in Figure 4A, cibacron was found to be a powerful blocker of ATP-induced currents, possibly more powerful than suramin. However, as shown in Figure 4B and 4C, cibacron also attenuated the voltage-induced outward potassium currents. Therefore, the effects of cibacron in vivo may be due to effects on both voltage-induced potassium currents and ATP-induced currents.

An additional ATP antagonist, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), was examined at the single cell level and found to block ATP-induced currents (Figure 5). The block was slow to develop, as indicated by the much larger block of ATP after washing out the PPADS. To date, this block of ATP has not accompanied by other effects of PPADS, such as effects on

voltage-induced potassium currents (Figure 5B and 5C). It appears that PPADS is a more potent ATP antagonist than either suramin or cibacron.

In summary, the only effects detected for both suramin and PPADS at the single cell level was a block of ATP-induced inward current. Cibacron, on the other hand, also affected voltage-induced potassium currents. Thus, the suppression of the cubic DPOAEs by cibacron, but not by suramin, possibly reflects the actions of cibacron on outward potassium currents.

4.2.3. The time varying amplitude change in the quadratic DPOAE (f_2-f_1).

After a period of silence, the quadratic DPOAE undergoes complex time varying changes in amplitude when monitored over minutes during continuous sound stimulation with moderate level primaries (Brown, 1988; Kirk and Johnstone, 1993; Kujawa et al. 1995, 1996; Lowe and Robertson, 1995). The typical shape of response recorded from guinea pig cochlea is shown in Figure 6. Initially, it was thought that the efferent nerves innervating the cochlea were responsible for these changes in amplitude. However, Lowe and Robertson (1995) demonstrated conclusively that the efferents do not contribute to the amplitude change. Similar conclusions were reached by Kujawa et al. (1995, 1996) who demonstrated that the amplitude change recorded from guinea pig was

not affected to a large extent by: TTX, a sodium channel antagonist; curare in concentrations that blocked the action of acetylcholine released by the efferents onto the OHCs; bicuculline, a GABA antagonist; and efferent sectioning. Thus it appears that neither the efferents or any other kind of nerve action underlies the amplitude change. On the other hand, the amplitude was dramatically affected by drugs active at calcium channels such as nimodipine and Bay K (Kujawa et al., 1996).

4.2.4. Effect of ATP antagonists on the time-varying amplitude change of the quadratic DPOAE.

Others suggested that the quadratic DPOAE is a more sensitive indicator of the set point of the cochlear amplifier (Frank and Kossl, 1996). Thus the quadratic DPOAE may be a more sensitive indicator of the role of the OHC-Deiters' cell complex in the function of the cochlear mechanics. Therefore, we extended our pharmacological studies of the quadratic amplitude change include the effects of ATP antagonists.

Figure 7 shows the effect of perfusing increasing concentrations of suramin through the perilymph compartment of guinea pig cochlea on the amplitude change in the quadratic DPOAE. Suramin blocked the decline in the amplitude of the quadratic DPOAE in a reversible manner without an effect on the initial starting value. Monitoring the amplitude of the quadratic DPOAE at various

primary intensities indicated that the suramin shifted the function to the left (Fig. 8). In contrast the amplitude growth function of the cubic DPOAE was not altered (Fig. 8).

When PPADS was tested in the same fashion as suramin, PPADS altered the amplitude change in a much more complex fashion than suramin (Fig. 9). Low concentrations of PPADS (< 0.10 mM) suppressed the initial value of the quadratic DPOAE at 60 dB primaries immediately after the silence period (Fig.9) with little effect on the overall shape of the change over time. Higher concentrations of PPADS (0.33 and 1 mM) induced a further suppression of the initial value and also reversed the decline, causing instead, a slow dramatic rise (Fig. 9). The subsequently recorded amplitude growth functions for the quadratic and cubic DPOAEs were shifted to the left (Fig. 10).

Thus there are differences between the effects of these two ATP antagonists. Suramin appears to solely abolish the decline in the quadratic DPOAE while PPADs both (1)suppresses the initial, post-silence value of the DPOAE and (2)abolishes, or reverses, the decline. The reason for these differences in action of the two drugs is presently unknown, however, it may be the fact that PPADS is a more potent blocker of ATP than suramin. Both PPADS and suramin are equally potent in inhibiting ectonucleotidases (41% inhibition at 100 μM; Ziganshin et al., 1995).

5. Additional criteria.

5.1 Release criterion (#3).

Evidence for the release criterion has been obtained from several sources. In the vestibular system, Bryant et al. (1987) demonstrated release of adenosine from the isolated semicircular canal in response to electrical stimulation. Since the authors made no attempt to block the degradation of any released ATP by ectonucleotidases, this adenosine may possibly represent an ATP breakdown product. If so, then Bryant et al. (1987) may well have demonstrated ATP release in response to depolarization of this hair cell containing organ.

Along similar lines, we have preliminary data to indicate that ATP release may be detected in effluent from the perilymph compartment. In an experiment carried out over ten years ago with Drs. Bryant and Guth, increased levels of adenosine were detected in perilymph compartment effluent of guinea pig cochlea in response to raised potassium levels (see Bryant et al., 1987). Again this adenosine may represent an ATP breakdown product. This experiment may be taken as very preliminary evidence that ATP is released from cochlear tissue in response to depolarization and that it may be detected in perilymph effluent. The cells of origin of the adenosine (and therefore most probably the ATP) are unknown since the high potassium may have induced release from stria cells as well as inner hair cells, outer hair cells, Deiters' cells, etc. In addition, the potassium may have caused non-specific swelling

of the cells with the resultant spilling of the cytoplasmic ATP into the perilymph. Therefore these experiments will have to be replicated to determine if the adenosine (or ATP) release was calcium dependent and thus vesicular, and not due to cells leaking the contents of their cytoplasm.

Recently, Wangemann (1996) and Wangemann and Marcus (1994) demonstrated the calcium dependent release of ATP from isolated gerbil organ of Corti. The cellular source of the ATP is unknown. Likewise the resolution was not able to determine the target cells of the ATP that was released. The origin of the endogenous ATP that acts on the cells in the organ of Corti remains to be determined.

5.2 Synthesis criterion (#4).

The presence of isozymes of creatine kinase in Deiters' cells suggests that the released ATP may come from Deiters' cells (Spicer and Schulte, 1992). These enzymes synthesize ATP. Whether this ATP is the source of the ATP released onto the receptors on Deiters' cells surface to modulate the mechanics of the organ of Corti remains to be shown.

5.3 Termination criterion (#5).

Breakdown of any released ATP will be rapid as demonstrated

by Vlajkovic et al. (1996) who presented evidence that ATP in perilymph is rapidly metabolized by ectonucleotidases to adenosine, adenosine 5'-diphosphate, and adenosine 5'-monophosphate. Therefore any released ATP will probably appear in perilymph effluent as one of these products. Thus the termination criterion (#5) appears to have been met, at least for ATP released into the perilymph compartment in the vicinity of the OHCs and Deiters' cells.

5.4 Receptor protein and mRNA criterion (#6)

To date only a single P2X receptor type mRNA has been identified in the cochlea of rat (Housley et al., 1995; Brandle et al., 1997). Guinea pig mRNA for P2X2 has also been found in the organ of Corti library (Parker et al., 1997). Thus the receptor protein and mRNA criterion (#6) is in the process of being met.

6. Summary and Model.

Currently, our model includes the endogenous release of ATP possibly from OHCs or Deiters' cells onto ATP receptors on Deiters' cells. This ATP may be released in response to a sound stimulus that depolarizes the OHC, inducing release of ATP from the OHC. Alternatively, sound may increase the concentration of potassium outside of Deiters' cells, inducing depolarization of Deiters' cells and subsequent release ATP from the Deiters' cells. The large concentration of the ATP synthesizing enzyme, creatine

kinase, in Deiters' cells suggests that the source of exogenous ATP is the Deiters' cells. The released ATP will induce an influx of calcium (plus soduim and potassium) into the Deiters' cell through the ATP activated ion channel. This calcium may alter the mechanical stiffness of the Deiters' cell to increase or decrease the tension applied by the Deiters' cell on the OHCs. This in turn may adjust the cochlear amplifier in the face of a changing sound stimulus over time.

Based on the effects of the ATP antagonists, suramin and PPADS, we speculate that the continuous time-varying amplitude change in the quadratic DPOAE may be a very sensitive measure of the effects of endogenous ATP acting in the above manner. We propose the following working model to explain the actions of the drugs. In this model, the magnitude of the quadratic DPOAE is determined by the amount of ATP acting at P2X2 receptors on Deiters' cells. The greater the amount of ATP acting on the receptors, then the larger this value. During silence, we propose that there is a basal level of ATP release. Upon turning on the primaries, the amount of ATP released is increased, producing the small increase in the quadratic DPOAE seen at about 1 min into the primary stimulation. The subsequent decline in the quadratic DPOAE during continuous primary stimulation is then thought to be due to depletion of ATP from the release site. In addition, it is assumed that released ATP is rapidly broken down into inactive products by ectonucleotidases.

In this model, PPADS, a more powerful ATP antagonist than suramin, decreases the initial value of the quadratic DPOAE due to block of ATP at receptors on Deiters' cells. The PPADS-induced inhibition of the ectonucleotidases (41% inhibition at 100 μM; Ziganshin et al., 1995), prevents the breakdown of ATP released in response to the presence of continuous primaries. The released ATP will accumulate in the extracellular space. This accumulated ATP will competitively displace PPADS from the receptors, allowing the ATP to act on the receptor. This receptor activation then reverses the decline in the quadratic DPOAE that occurs over time in the presence of continuous primaries.

In contrast, suramin is a weaker receptor antagonist, but equipotent with PPADS in inhibiting ectonucleotidases (41% inhibition at 100 μM; Ziganshin et al., 1995). Any block of receptor by the suramin molecule is probably displaced by ATP accumulating in the extracellular space due to an inhibition of breakdown by the enzyme. Also, according to our single cell data, any accumulated ATP may be potentiated by suramin at the P2X2 receptor. This would explain the lack of effect of suramin on the initial value of the quadratic DPOAE. In addition, by blocking the breakdown of ATP, suramin would reverse the decline in the quadratic DPOAE.

This model does not take into consideration the possible location of metabotropic ATP receptors on OHCs, Deiters' cells or

on Hensen's cells. The latter which are connected to Deiters' cells by gap junctions have ionotropic ATP receptors. In addition, many of the actions of the drugs that are described may be due to these other receptors. Therefore, the validity of this model will be determined by future research. However, the proposed model does explain the action of the drugs and allows for the design of further experiments.

Acknowledgments

The authors wishes to thank Maureen Fallon for drawing the illustrations and Latasha Bright, and Christopher LeBlanc for help. This work was supported in part by research grant numbers DC 00722-05 and DC00379 from the National Institute on Deafness and Other Communication Disorders, National Institutes of Health, and DAMD 17-93-V-3013, Kam's fund for Hearing Research, and the Louisiana Lions Eye Foundation.

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Figure legends.

Fig. 1. Effect of ATP and ATP-γ-S on cubic ($2f_1$-f_2) DPOAE responses recorded from the external ear canal as a function of stimulus intensity. Shown in each panel are amplitude growth functions recorded after pre-drug artificial perilymph perfusions (AP2) and after perfusion with increasing concentrations of ATP and ATP-γ-S. Data are represented as mans ± S.E. across 5 animals. From Kujawa et al.(1994a).

Fig. 2. Effect of suramin and cibacron on the cubic ($2f_1$-f_2) DPOAE as a function of stimulus intensity. Shown are amplitude growth functions recorded after pre-drug artificial perilymph perfusions (AP2) and after perfusion with increasing concentrations of suramin and cibacron. Data are represented as means ± S.E. across 5 animals for each drug. Taken from Kujawa et al. (1994b).

Fig. 3. Suramin blocked the effects of ATP and had no effect on voltage induced currents in a Deiters' cell. A: ATP (5 μM) induced currents are suppressed by suramin (100 μM). After washing out the suramin, the ATP response was greater than control. The cell was voltage clamped at -80 mV. B: Current voltage (I-V) relationships recorded from a Deiters' cell in the absence and presence of 100 μM suramin. The voltage command was stepped from -100 mV to +60 mV. C: I-V plots were constructed from the steady state (Ss) and

instantaneous (In) values in B.

Fig. 4. Cibacron suppressed both the ATP-induced currents and voltage-induced currents in a Deiters' cell. A: Cibacron (100 μM) suppressed the ATP (5 μM)-induced current. The cell was voltage clamped at -80 mV. B: Current-voltage (I-V) relationships recorded from a Deiters' cell in the absence and presence of 100 μM cibacron. The voltage command was stepped from -100 mV to +60 mV. C: I-V plots were constructed from the steady state (Ss) and instantaneous (In) values in B.

Fig. 5. PPADS suppressed ATP-evoked currents with no effect on voltage-induced currents in a Deiters' cell. A: PPADS (10 μM) suppressed the ATP (5 μM)-evoked currents with a delay. The delay is observed as a greater blockade after washing out the PPADS than the amount of blockade when the PPADS was simultaneously applied with the ATP. The cell was voltage clamped at -80 mV. B: Current voltage (I-V) relationships recorded from a Deiters' cell in the absence and presence of 10 μM PPADS. The voltage command was stepped from -100 mV to +60 mV. C: I-V plots were constructed from the steady state (Ss) and instantaneous (In) values in B.

Fig. 6. A typical example of the effect of continuous primary stimulation on cubic (f_2-f_1) DPOAE amplitude recorded from the ear canal immediately following a 15 min perfusion of the perilymph compartment with a second artificial perilymph (AP2) during which

time the primaries were turned off and the animal kept in a sound attenuated environment. Each data point represents a 10-spectra average and required 5 s to complete. The break in the response amplitude trace (C-D) represents 1 min with no primary stimulation. Points A - F thus identified in each trace were used to calculate amplitude changes.

One hundred consecutive 10-spectra averages of distortion product amplitude were obtained during continuous primary stimulation (f_1 = 6.25 kHz, f_2 = 7.5 kHz, L1 = L2 = 60 dB SPL). Each of these averages required approximately 5 s to complete for a total of 500 s (8.3 min) of stimulation. The primary tones were then simultaneously turned off and there was a 1 min rest from primary stimulation. Following this rest, the primaries were re-introduced and 40 consecutive 10-spectra averages of distortion product amplitude were obtained (total time approximately 200 s or 3.3 min of stimulation).

Fig. 7. Suramin attenuates the decrease in the quadratic (f_2-f_1) DPOAE amplitude induced by continuous primary stimulation. Shown are functions recorded in a typical animal after 15 min perfusions of pre-drug artificial perilymph perfusions (AP2), with increasing concentrations of suramin (0.033 - 1 mM), and a wash.

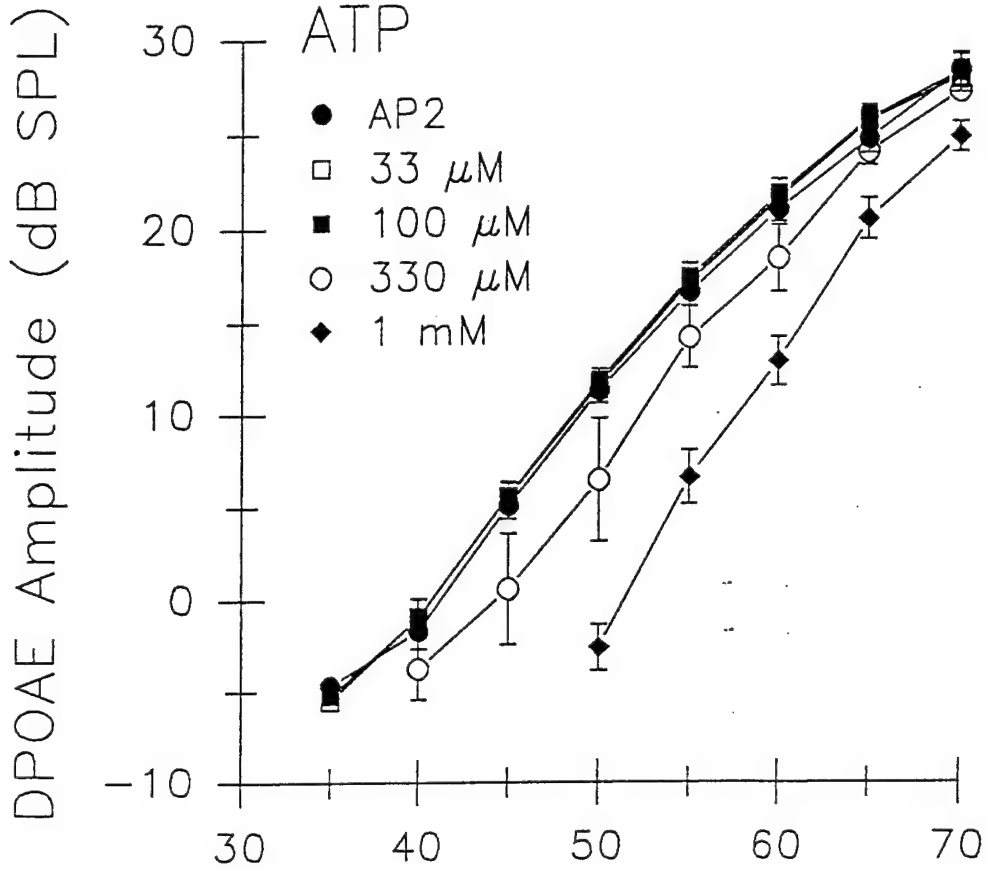
One hundred consecutive 5-spectra averages of distortion product amplitude were obtained during continuous primary stimulation (f_1 = 6.25 kHz, f_2 = 7.5 kHz, L1 = L2 = 60 dB SPL).

Each of these averages required approximately 3.5 s to complete for a total of 350 s (5.83 min) of stimulation. The primary tones were then simultaneously turned off and there was a 1 min rest from primary stimulation. Following this rest, the primaries were re-introduced and 40 consecutive 5-spectra averages of distortion product amplitude were obtained (total time approximately 140 s or 2.3 min of stimulation).

Fig. 8. Suramin enhances the amplitude growth function for the quadratic (f_2-f_1) but not the cubic ($2f_1$-f_2) DPOAE. Shown are values obtained in a typical example following perfusion of the perilymph compartment with a the second control artificial perilymph perfusion (AP2), suramin (100 μM), and a wash with AP (wash 1). The dashed line in each panel represents the average value of the noise floor.

Fig. 9. PPADS induces complex changes in the overall shape of the the quadratic (f_2 -f_1) DPOAE amplitude induced by continuous primary stimulation. Response amplitude as recorded following 15 min perfusions (in silence)of the perilymph compartment with the second artifical perilymph (AP2), increasing concentrations of PPADS (0.033 - 1.0 mM) and a wash with AP (wash). Data collected as described in the legend for Fig. 6.

Fig 10. PPADS enhances the growth functions for the quadratic ($f_2 - f_1$) and the cubic ($2f_1 - f_2$) DPOAE. Shown are values obtained following collection of the data shown in Fig. 9 for the second control perfusion (AP2), PPADS (1 mM), and a wash with AP. The dashed line in each panel represents the average value of the noise floor.


ATP
AP2
33 μM
100 μM
330 μM
1 mM
DPOAE Amplitude (dB SPL)
30
20
10
0
−10
30
40
50
60
70

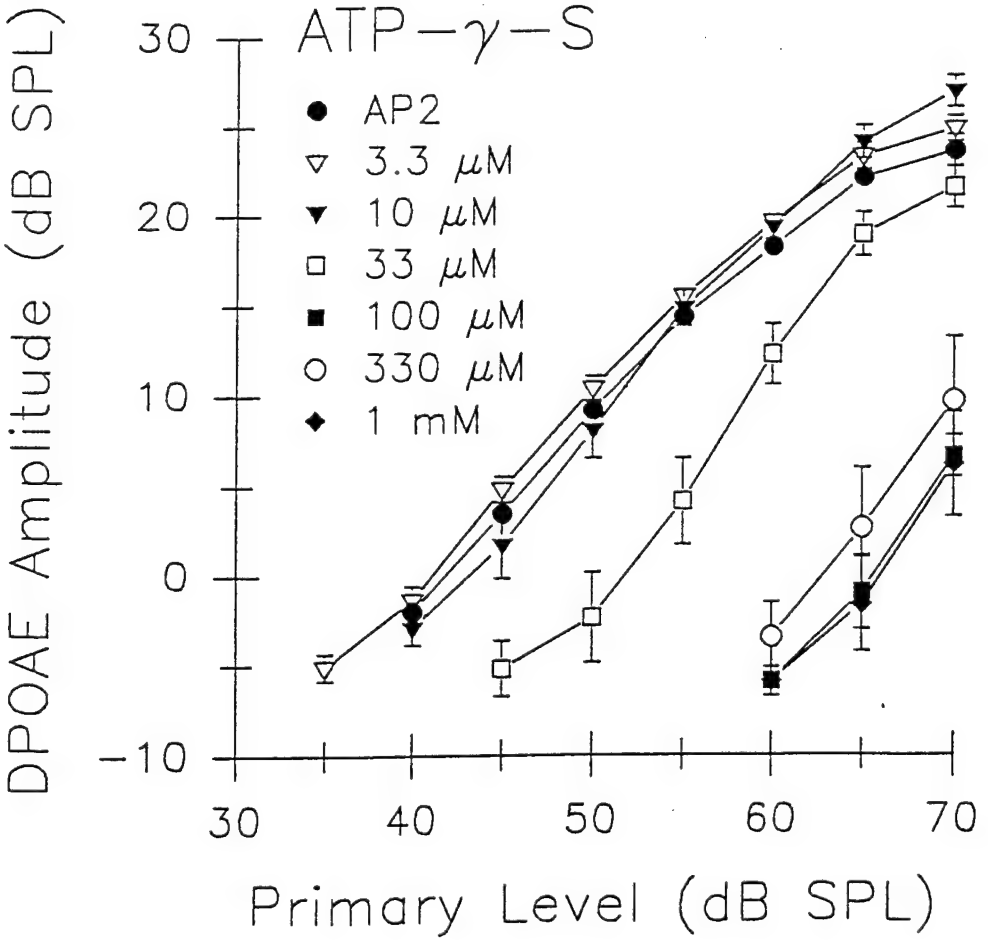
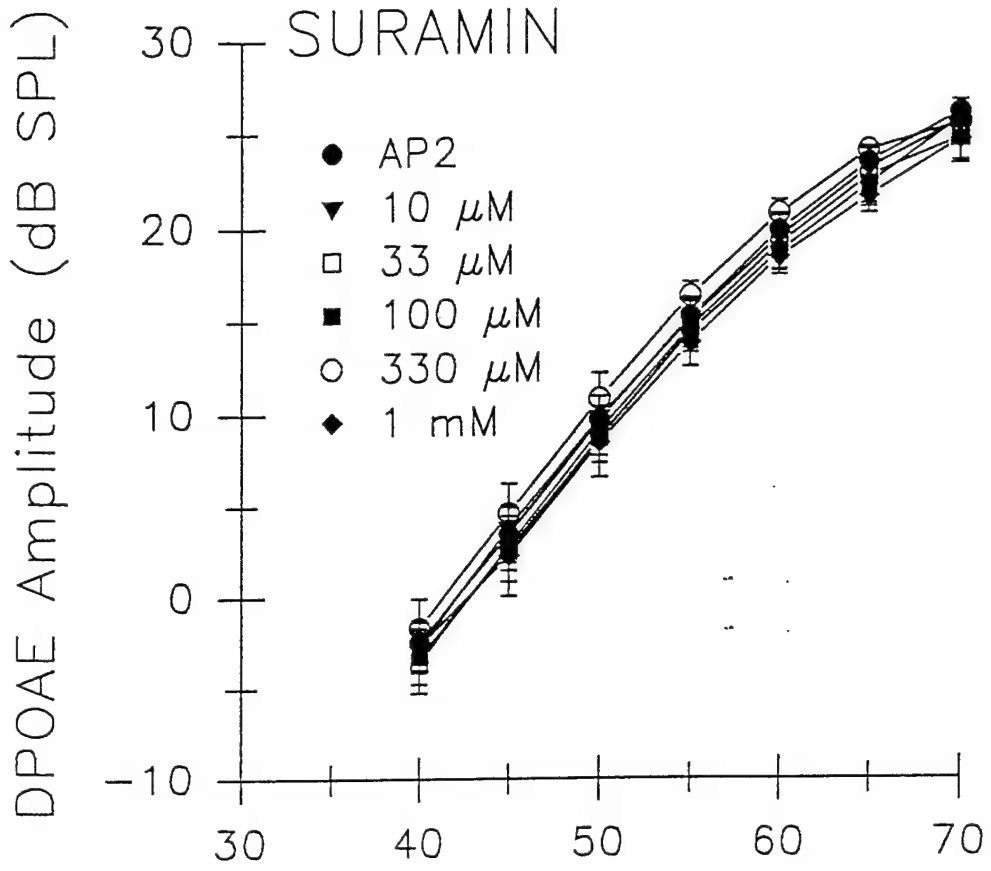

ATP−γ−S
AP2
3.3 μM
10 μM
33 μM
100 μM
330 μM
1 mM
DPOAE Amplitude (dB SPL)
30
20
10
0
−10
30
40
50
60
70
Primary Level (dB SPL)

Figure #1


SURAMIN
AP2
10 μM
33 μM
100 μM
330 μM
1 mM
DPOAE Amplitude (dB SPL)
30
20
10
0
−10
30
40
50
60
70

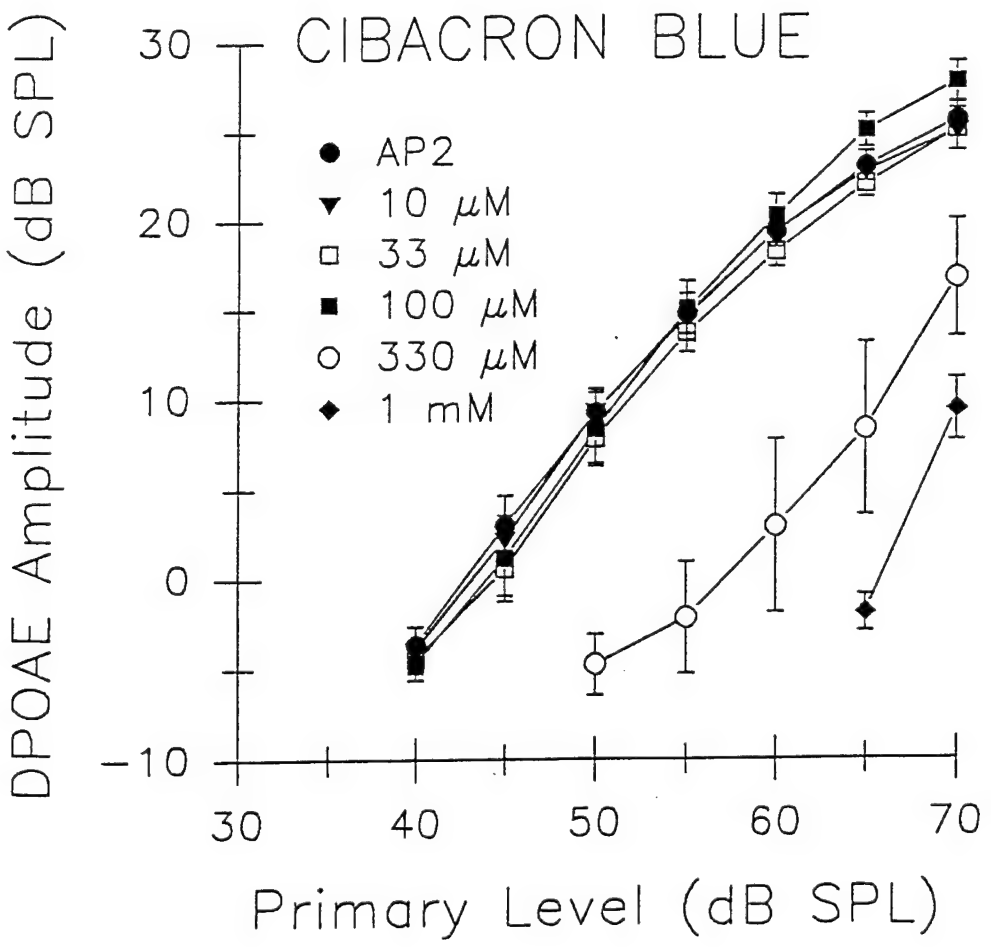
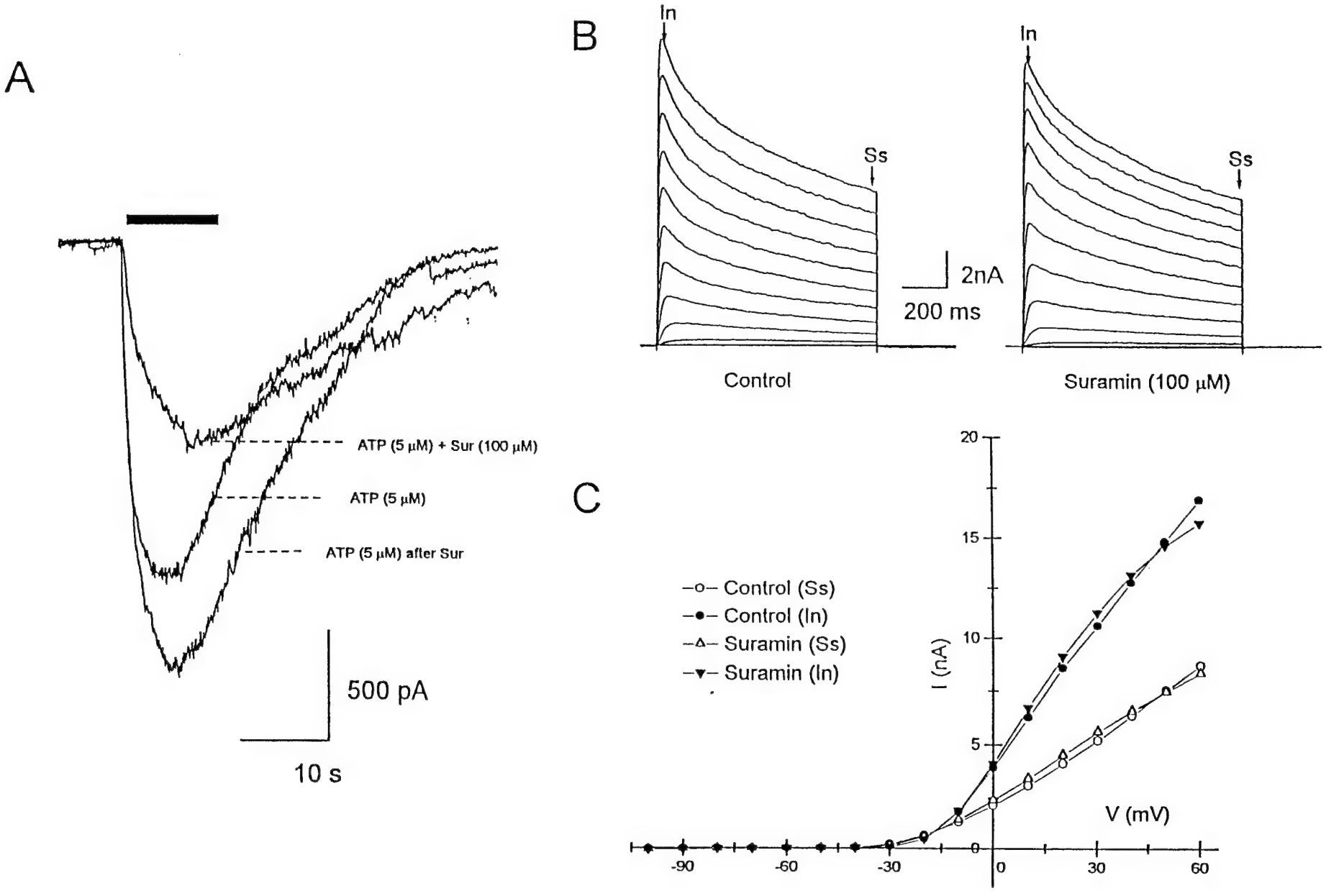
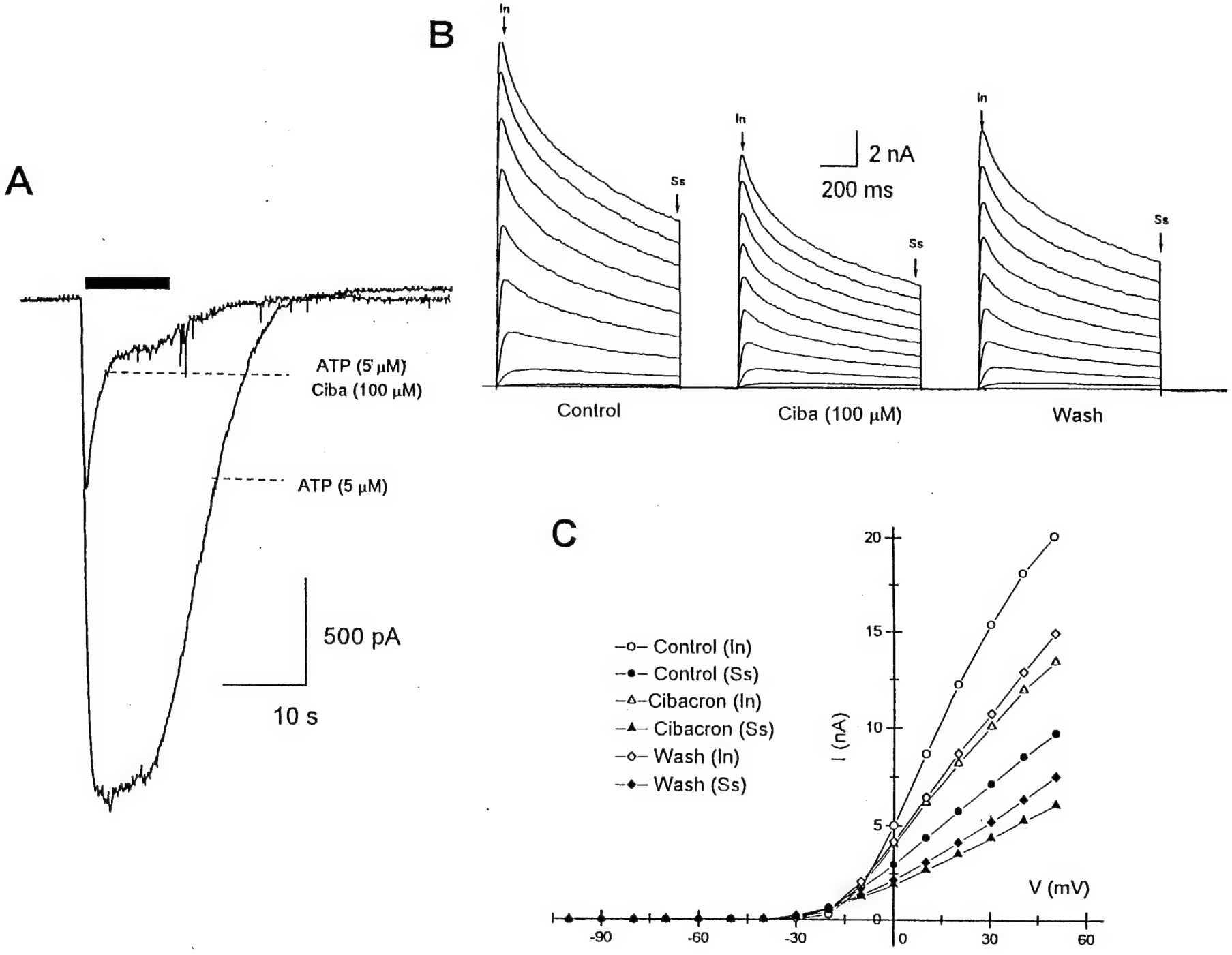

CIBACRON BLUE
AP2
10 μM
33 μM
100 μM
330 μM
1 mM
DPOAE Amplitude (dB SPL)
30
20
10
0
−10
30
40
50
60
70
Primary Level (dB SPL)

Figure #2


A
ATP (5 μM) + Sur (100 μM)
ATP (5 μM)
ATP (5 μM) after Sur
500 pA
10 s
B
In
Ss
In
Ss
2nA
200 ms
Control
Suramin (100 μM)
C
Control (Ss)
Control (In)
Suramin (Ss)
Suramin (In)
I (nA)
V (mV)
20
15
10
5
0
-90
-60
-30
0
30
60


A
ATP (5 μM)
Ciba (100 μM)
ATP (5 μM)
500 pA
10 s
B
In
Ss
2 nA
200 ms
Control
Ciba (100 μM)
Wash
C
Control (In)
Control (Ss)
Cibacron (In)
Cibacron (Ss)
Wash (In)
Wash (Ss)
I (nA)
V (mV)
20
15
10
5
0
-90
-60
-30
0
30
60

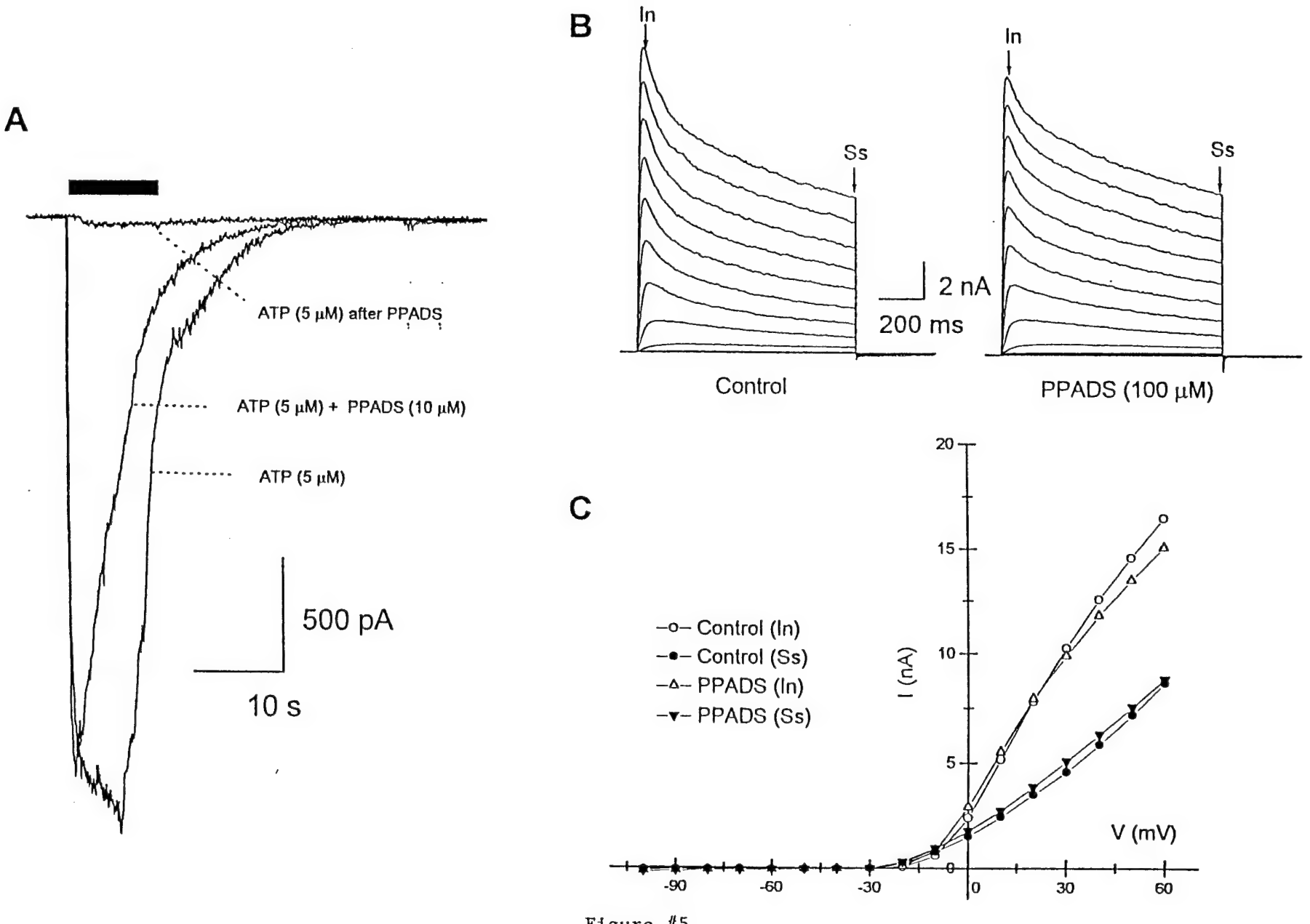

A
ATP (5 μM) after PPADS
ATP (5 μM) + PPADS (10 μM)
ATP (5 μM)
500 pA
10 s
B
In
Ss
In
Ss
2 nA
200 ms
Control
PPADS (100 μM)
C
Control (In)
Control (Ss)
PPADS (In)
PPADS (Ss)
I (nA)
V (mV)
20
15
10
5
0
-90
-60
-30
0
30
60

Figure #5

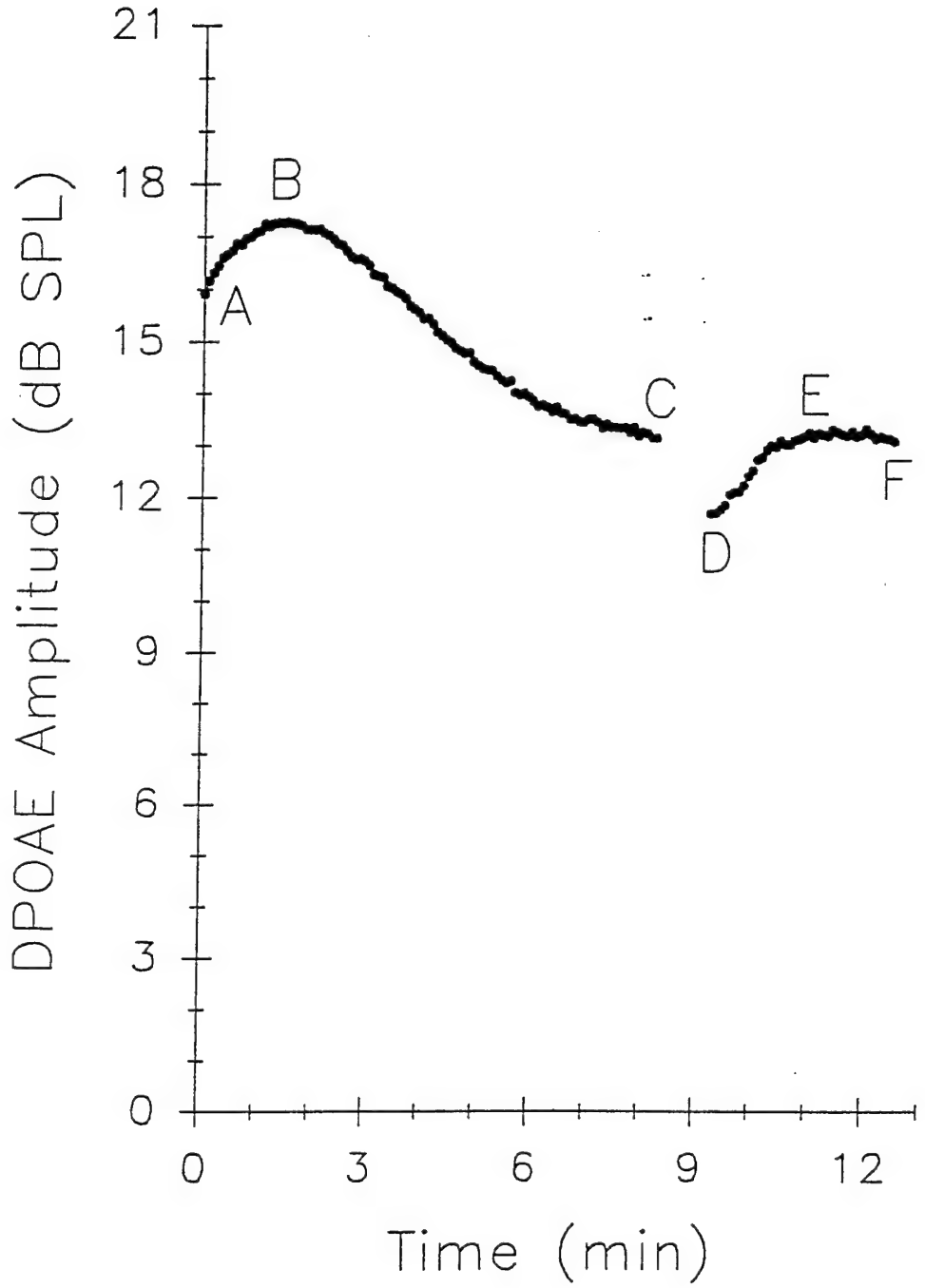
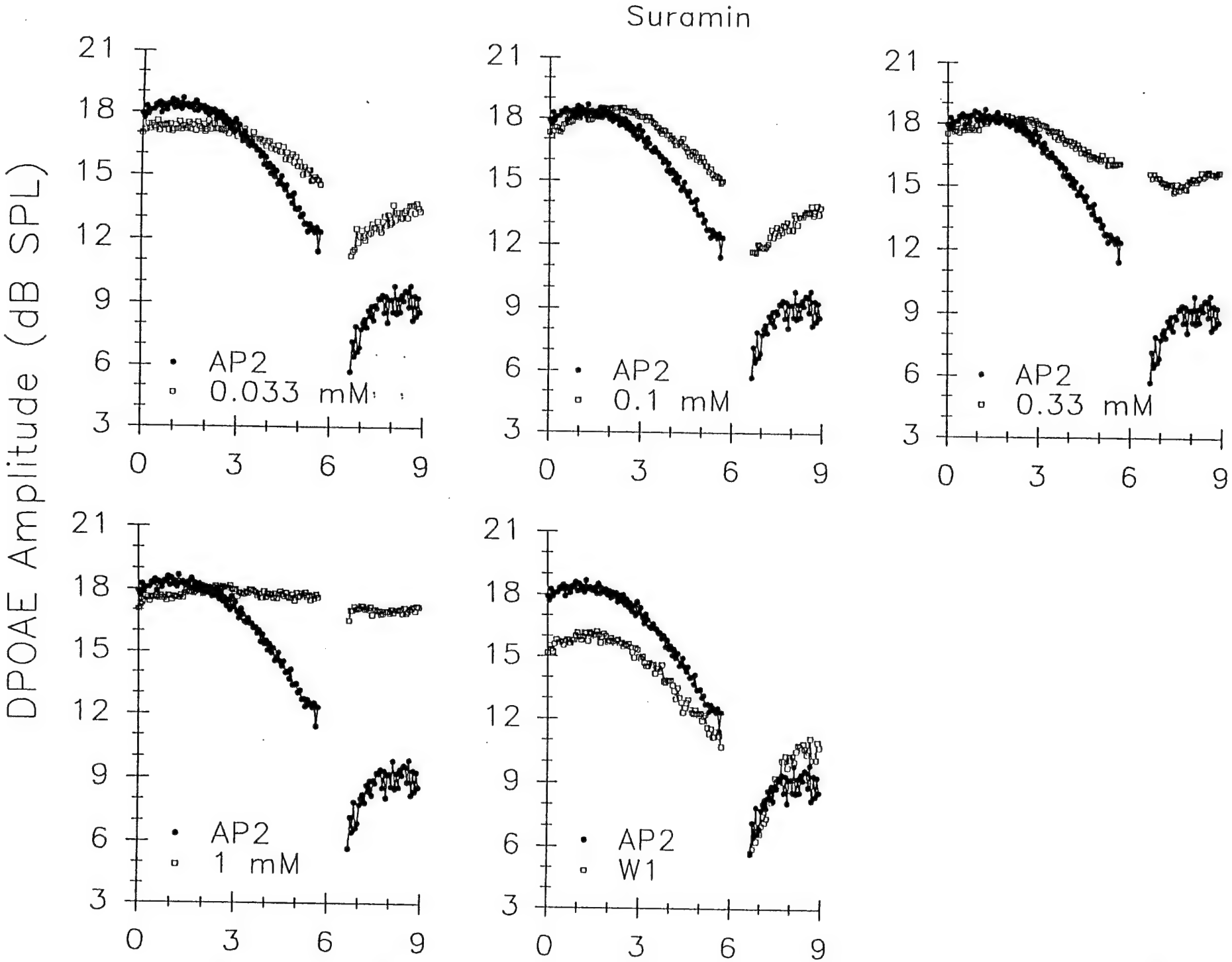

DPOAE Amplitude (dB SPL)
21
18
15
12
9
6
3
0
A
B
C
D
E
F
0
3
6
9
12
Time (min)

Figure #6


Suramin
DPOAE Amplitude (dB SPL)
21
18
15
12
9
6
3
0
3
6
9
AP2
0.033 mM
AP2
0.1 mM
AP2
0.33 mM
AP2
1 mM
AP2
W1

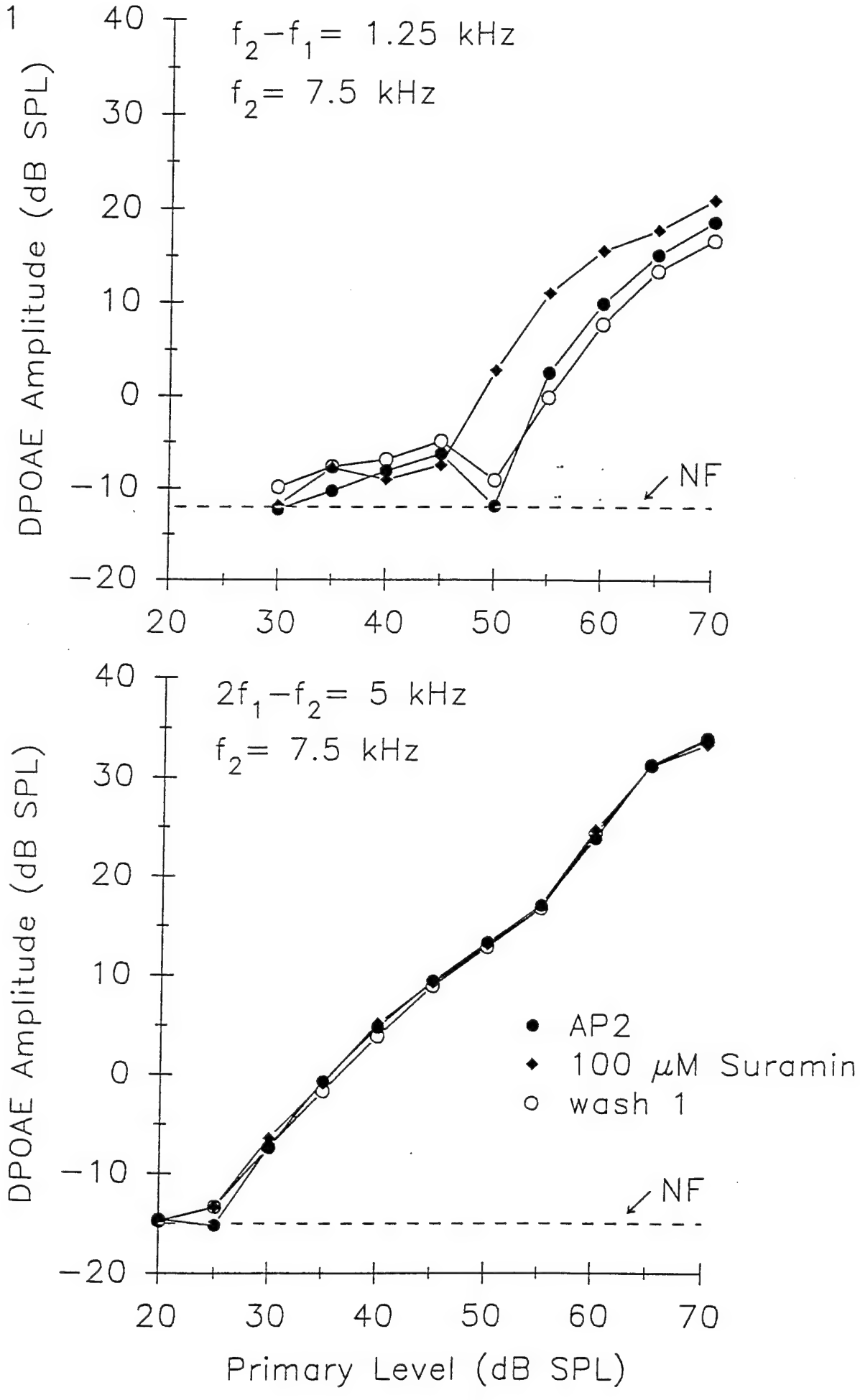
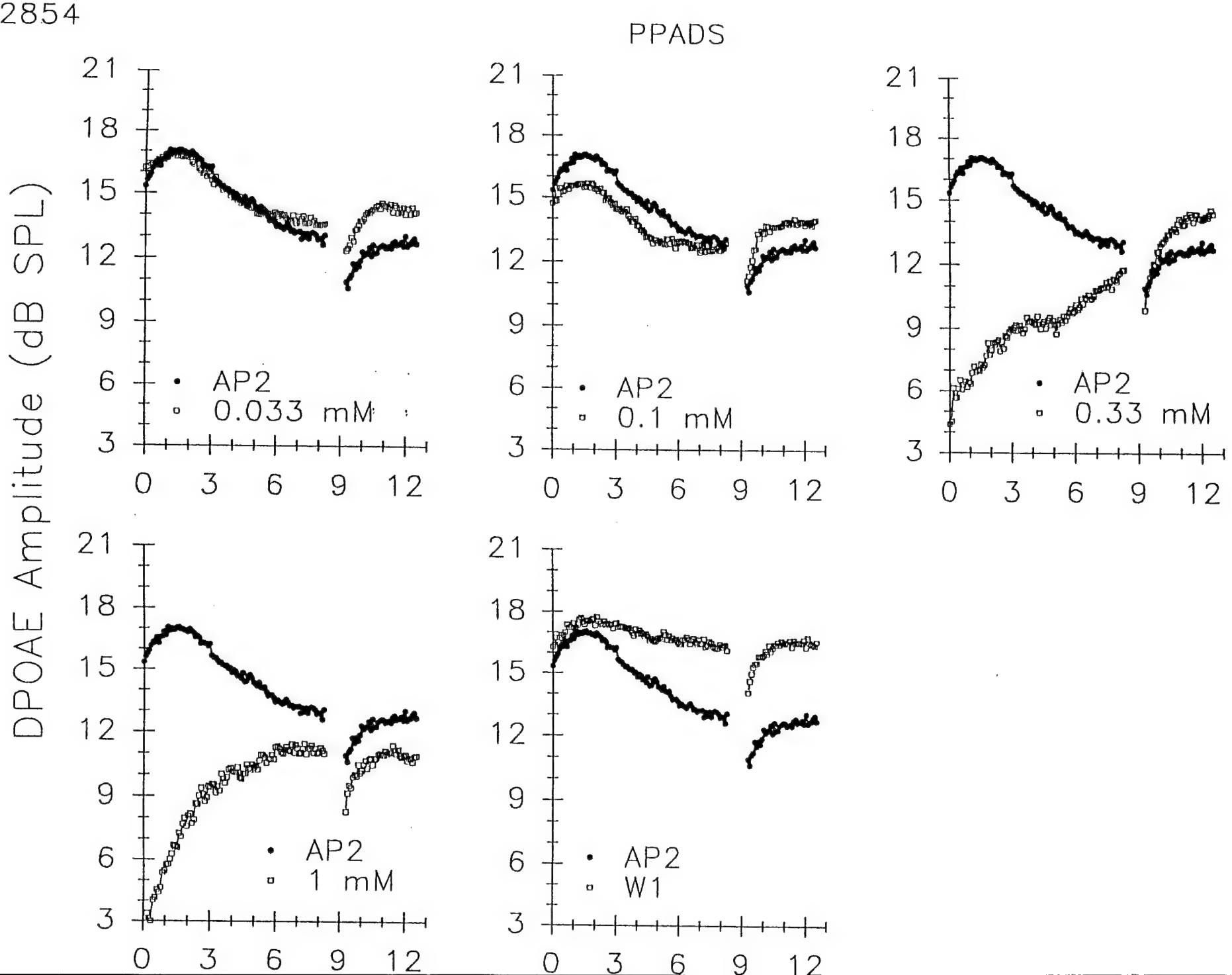

f_2-f_1= 1.25 kHz
f_2= 7.5 kHz
DPOAE Amplitude (dB SPL)
NF
$2f_1-f_2$= 5 kHz
f_2= 7.5 kHz
AP2
100 μM Suramin
wash 1
Primary Level (dB SPL)

Figure #8

2854


PPADS
DPOAE Amplitude (dB SPL)
21
18
15
12
9
6
3
0 3 6 9 12
AP2
0.033 mM
AP2
0.1 mM
AP2
0.33 mM
AP2
1 mM
AP2
W1

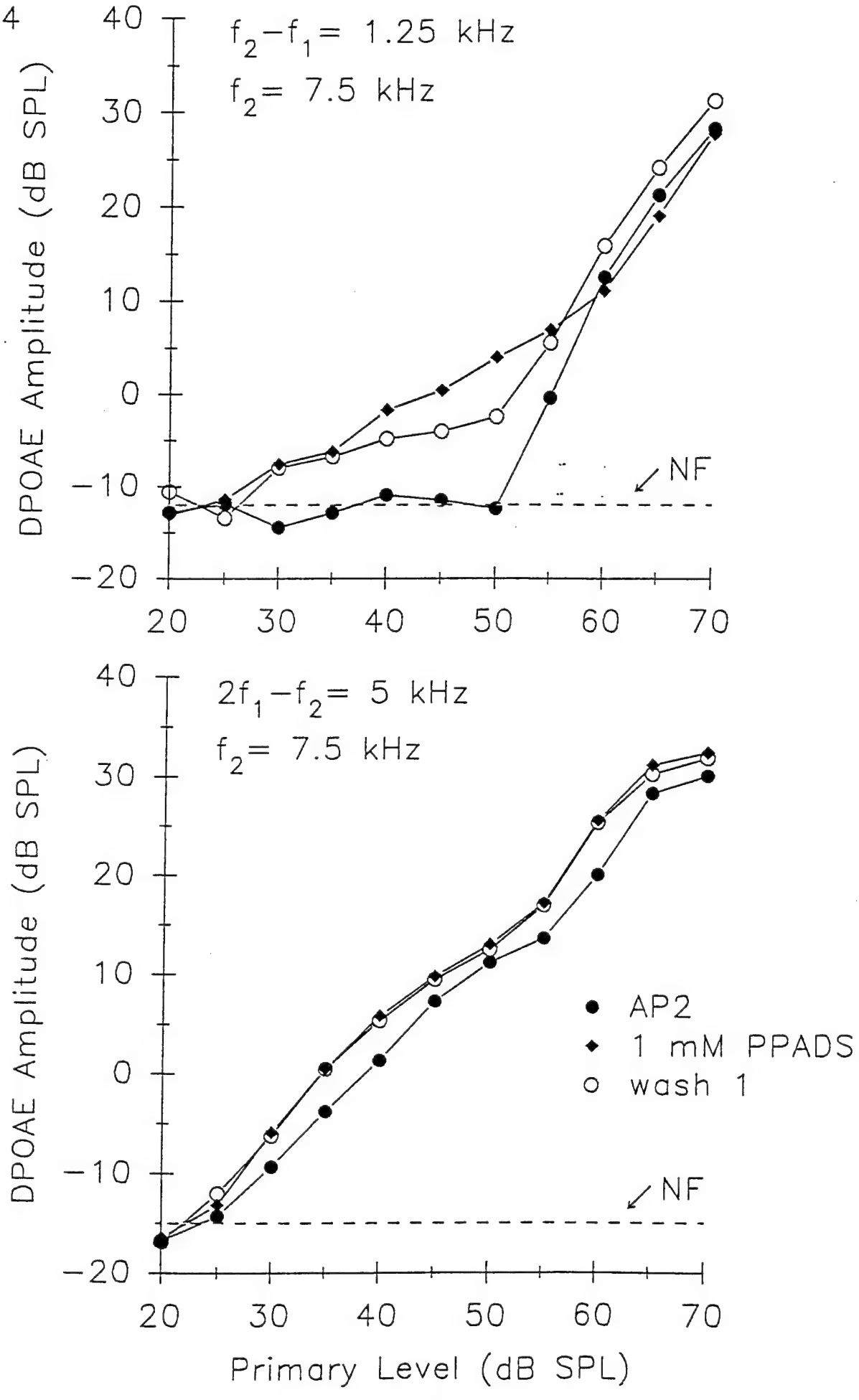

f_2-f_1= 1.25 kHz
f_2= 7.5 kHz
DPOAE Amplitude (dB SPL)
NF
$2f_1-f_2$= 5 kHz
f_2= 7.5 kHz
AP2
1 mM PPADS
wash 1
Primary Level (dB SPL)

Figure #10

Appendix #12
DAMD17-93-V-3013
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Hearing Research 87 (1995) 96–103

HEARING RESEARCH

Binaural noise suppresses linear click-evoked otoacoustic emissions more than ipsilateral or contralateral noise

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Received 7 November 1994; revised 25 March 1995; accepted 4 April 1995

Abstract

We studied the efferent suppression of click-evoked otoacoustic emissions with 65 dB SPL of white noise presented to left, right, or sometimes both, ears for 408 ms. Each burst of noise preceded a series of four unipolar 80 μs 65 dB peak Sound Pressure clicks, presented to the left ear only. The first click of the four-click group followed the end of the noise by either 1, 2, 5, 10, 20, 50, 100 or 200 ms; each subsequent click was offset by 20 additional ms via an ILO88 system with special programming modifications. Conditions were alternated so that a 'without noise' condition preceded a 'with noise' condition for three repetitions of 600 clicks per trial. Seven subjects with normal hearing participated in the study, and three of the seven participated in a test-retest reliability study. Results showed the greatest suppression followed binaural stimulation ending within one to five ms of the first click in the pulse train. Somewhat less suppression was seen following ipsilateral stimulation. The least amount of suppression was seen following contralateral stimulation, suggesting that previous research using contralateral stimulation may underestimate efferent effects. We saw no effects when the end of the noise was 100 ms or more away from the beginning of the click train.

Keywords: Otoacoustic emissions; Efferent suppression; Binaural; Ipsilateral; Contralateral; Forward masking

1. Introduction

The medial olivocochlear system suppresses segments of outer hair cell activity when activated either contralaterally, ipsilaterally or bilaterally with an auditory stimulus of sufficient duration (Warr et al., 1986; Warr and Guinan, 1978; Puel and Rebillard, 1990; Liberman, 1989; Kujawa et al., 1993,1994).

Previous experiments on suppressing otoacoustic emissions in humans have focused mostly on the suppressive effects of continuous contralateral stimulation (e.g., Collet et al., 1990; Ryan et al., 1991; Berlin et al., 1993a,b,1995).

Kevanishvili et al. (1992) and Gobsch et al. (1992) studied forward masking of emissions in an attempt to relate the detection of hair cell suppression to perceptual masking. They used either unipolar clicks or 1000 Hz tone bursts, with time separations of 5 to 200 ms and masker durations of 6 and 50 ms; they saw little ipsilateral suppressive effect of the low level maskers on emission amplitude when judged subjectively by three independent judges.

Henson et al. (1994) offered a comprehensive summary of efferent contralateral effects on otoacoustic emissions. He noted that most of the published work showed suppression or reduction of emissions during contralateral acoustic or electrical stimulation; however, some workers, notably Brown and Norton (1990), and Plinkert and Lennarz (1992), reported an occasional increase (as well as decrease) in emission amplitude as a result of contralateral stimulation.

In this work we presented unipolar clicks to seven normal hearing human subjects to show the suppressive effects of low level binaural, ipsilateral, and contralateral white noise (408 ms in duration) in a forward masking paradigm. We used a proprietary analysis system (Wen et al., 1993) to record differences of as much as 7 dB between control and experimental traces that were not immediately apparent to casual observation.

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SSDI 0378-5955(95)00082-8

2. Methods

The ILO88 system is currently widely used for the ecording of transient evoked emissions (Kemp et al., 989). In the default so-called non-linear condition three 30 dB peak sound pressure pulses are presented in one polarity while a fourth pulse is presented out of phase with he first three and at 10 dB greater intensity. The resulting echo represents the difference between the sum of the hree echoes elicited at 80 dB and the one out-of-phase echo elicited by the 90 dB pulse; the strategy is designed o cancel any artifactual ringing which could be mistaken for hair cell echoes.

Suppression is evident, but not robust, with these de-fault conditions (Berlin et al., 1993b). In contrast, efferent effects are more readily seen at low intensities than at high and with linear rather than non-linear click trains (Collet et al., 1990; Hood et al., 1994); therefore, we designed our experiments around low level linear clicks and noises.

One of us (D.K.) supplied the program for an ILO88 system to control the temporal interval between the offset of a duration-controlled noise stimulus and the onset of a 4-click train. We selected a 408 ms duration noise based on the work of Liberman (1989) and Huang et al. (1994) to maximize the likelihood of activating the efferent system. The emission-evoking 80 μs clicks were all 65 dB peak sound pressure and all of the same polarity. The first click of the 4-click group followed the end of the noise by either 1, 2, 5, 10, 20, 50, 100, or 200 ms, whereas the final 3 clicks followed the first click by successive increments of 20 ms. Conditions were alternated so that a 'without noise' condition preceded a 'with noise' condition for three complete trials. The order of ipsilateral vs. contralateral vs. bilateral presentation of the noise was counterbalanced among subjects for the 24 different listening conditions. Three subjects were retested under conditions where the first of the 4 clicks began 1 ms after the termination of the noise and each subsequent click was offset by 20 ms.

Each set of control vs. experimental data consisted of the mean of three 'without noise' trials, and three 'with noise' trials, compared to one another using two separate quantification systems. In one case we compared the mean echoes to each other using the Kemp aggregate echo level number, which we called 'dB-ILO'. This number represents the overall spectral amplitude of the echoes averaged by the ILO88 system over a 20.48 ms window. The second method used the Kresge Echomaster system (Wen et al., 1993) which allows custom designed amplitude, time and frequency comparisons between means of control and experimental conditions. We chose to quantify the RMS differences between the echoes in two ms segments and labeled this number 'dB-K' to differentiate it from the overall RMS number available from the ILO88 system. The Kresge Echomaster system also allowed us to make temporal comparison of differences between segments of the control and experimental echoes in 40 μs steps (See Table 1 later for examples).

3. Results

The binaural noise condition generated 1.5 to 2 dB-ILO of emission suppression when the noise preceded the first click in the train by one to twenty ms. Thereafter the suppressive effects decreased as time-separation increased.

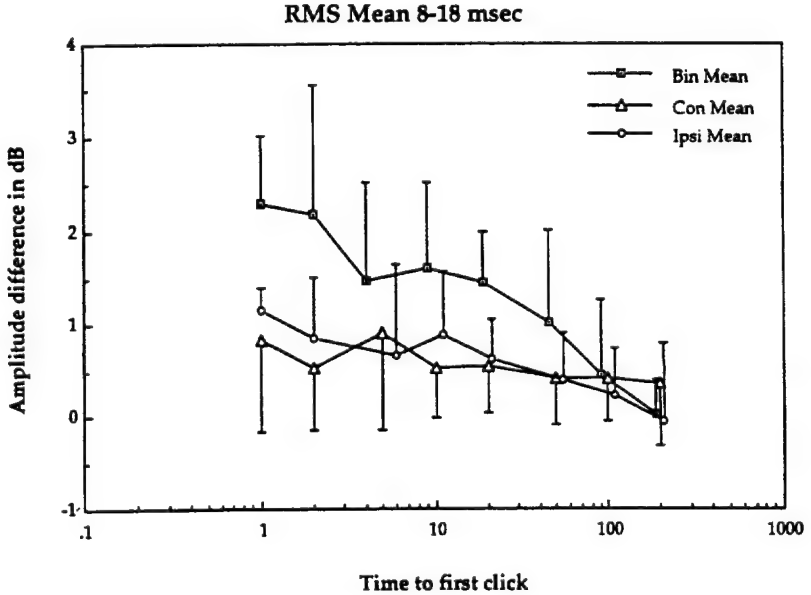


Fig. 1. The effects of 408 msecs of binaural, contralateral, or ipsilateral noise preceding a left ear click train in which the first click started at 1, 2, 5, 10, 50, 100, and 200 msecs after the end of the noise burst. Each of the subsequent clicks was offset by an additional 20 msecs. The mean and 1 Standard Deviation are presented using the amplitude difference data between 8–18 msecs from the Kresge Echomaster (KEM) 3.0 program.

The amount of suppression to binaural noise shown in Fig. 1 is 2.5 to 4 dB-K between 8 and 18 ms after the click stimulation, a zone in which the majority of suppression is seen. The values for ipsilateral and contralateral suppression are respectively smaller in this forward masking paradigm. Fig. 2 compares suppressive effects of binaural, ipsilateral and contralateral noise at 1, 10, 50 and 200 ms time separations between the end of the noise and start of the first click. The suppression is expressed in dB-K.

Fig. 3 offers another view of the same data. Here we compare the data in dB-K over the 4 to 20 ms period of the KEM 3.1 program analysis, showing the binaural data only when the noise terminates 1, 5, 20, and 200 ms before the first click. Then in the companion figure we show similar binaural data for time-separations of 2, 10, 50 and 100 ms between the end of the noise and the beginning of the first click. Again three primary trends are evident:

1. The shorter the time-separation between the end of the noise and first click, the greater the suppressive effect ($F = 6.44$; $df = 7,42$; $P < 0.001$). Duncan's Range Test showed that 1 ms time separation is most effective. There were no significant differences between 2, 5, 10, and 20 ms of separation. Thereafter separations of 50, 100, and 200 ms all differed significantly from one another.
2. Suppression of 2.5 to 3.5 dB-K takes place in the 8–18 ms zone after click onset ($F = 10.37$; $df = 8,48$; $P < 0.001$). Duncan's range Test showed that the greatest effects occur between 18 and 20 ms. No significant differences were seen between 10 and 18 ms, while the least suppression was seen in the 2–8 ms ranges.
3. Binaural noise generates more efferent suppression than either ipsilateral or contralateral noise ($F = 11.43$; $df = 2,12$; $P < 0.005$). Duncan's Range Test showed that, while binaural noise generated the most suppression, ipsilateral and contralateral stimulation did not differ from one another in their suppressive abilities, although they did exert significant amounts of suppression. We also observed that the sum of ipsilateral and contralateral suppression was within 0.8 of a dB of the suppression generated by binaural noise as was predicted by Kirk and Johnstone (1993).

Spectral analysis through a Hanning window available in the KEM 3.1 program showed a gradual shift in the largest spectral difference (at 2344 Hz) from 6.328 dB of binaural-noise-induced-suppression when the first click

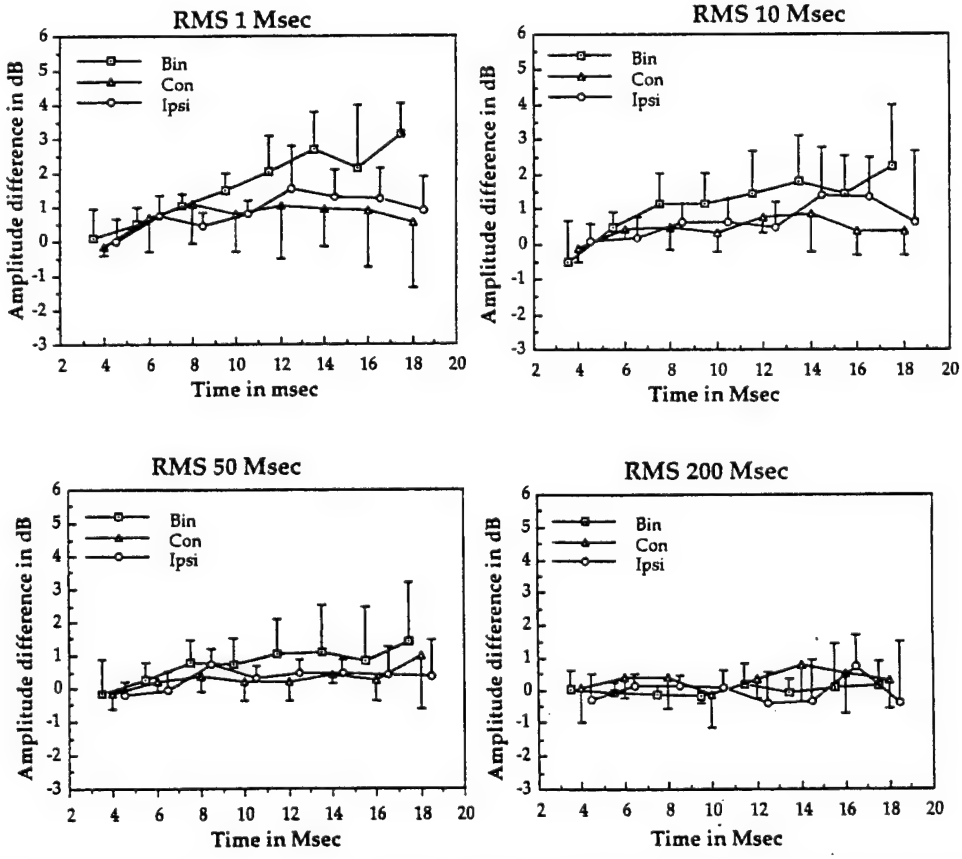


Fig. 2. The emission-suppressing effects of binaural, ipsilateral and contralateral noise. Data, expressed in db-K, are grouped by time intervals in which the noise preceded the first of four click-evoked emission by either 1, 10, 50 or 200 msecs.

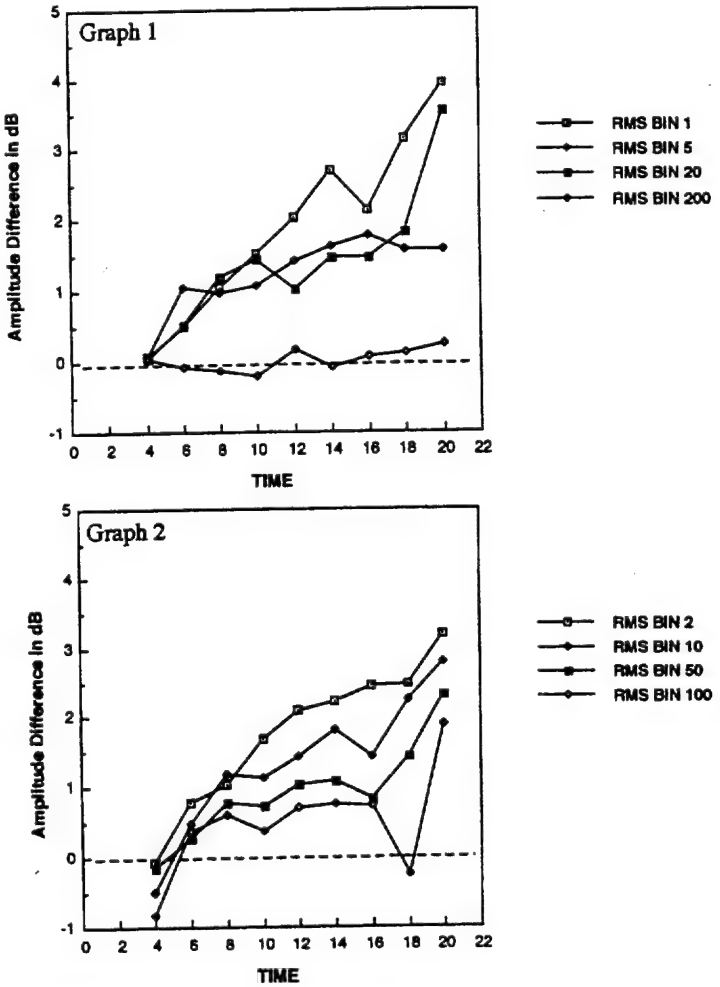
)wed the noise by only one ms, dropping to 2.267 dB
)0 ms time separation, and then down to 0.394 dB at
ms time separation for one representative subject
).). Other subjects showed qualitatively similar sup-
sion in spectral zones between 1000 and 3000 Hz.
Ve checked test-retest reliability for three subjects.
a for one subject are shown in Fig. 4. Group analysis
ws reliability as high as 0.62 for the binaural data at 1
time-separation but only 0.13 for the ipsilateral data
0.046 for the contralateral data. Reliability in general
as the time separation between the end of the noise and
onset of the click increased. This observation suggests
the binaural effects are clearly more robust and more
able than either ipsilateral or contralateral stimulation
his forward masking paradigm with humans.

Discussion

There are two major efferent systems which can affect emissions reaching the recording microphone: the middle ear muscle reflex efferent motor loop, and the olivocochlear efferents. The 80 μs click stimuli in this experiment were just detectable by our normal subjects at 38 dB peak sound pressure. Thus, since the clicks used in the experiment were presented at 65 dB peak sound pressure, they were only at 27 dB HL, a level far too faint to evoke a clinically measurable middle ear muscle reflex. The threshold for the noise was 22 dB SPL; therefore, the 65 dB SPL noise was only 43 dB HL, also a level far too low to elicit a clinical middle ear muscle reflex.

The suppressive effects are actually largest when the clicks and the noise are at lower intensities. We know this from separate work completed after this present experiment had started, which showed us that the suppressive effects of contralateral noise were largest when the clicks were at 55 dB peak sound pressure; the relative effects diminished when either the noise or the clicks were presented at higher intensities (Hood et al., 1994), a phenomenon also reported by Collet et al. (1990).

Finally, studies of people with no middle ear muscle function (e.g., Collet et al., 1990; Berlin et al., 1993a) all suggest that the middle ear muscle reflex did not partici-



. 3. Shows binaural noise suppression data for 1, 5, 20 and 200 msecs of time-separation between the end of the noise and the beginning of the first of r clicks. The companion figure shows the data for 2, 10, 50 and 100 msecs. Data are expressed in db-K.

pate in this experiment, although of course it is impossible to completely rule out subclinical middle ear muscle contraction in any one subject.

Still another potential confounding problem in such experiments is acoustic crosstalk. If standard ear phones such as TDH-39 with MX 41-AR cushions had been used, crossover by bone conduction or even partly by air conduction could conceivably take place at levels as low as 40 dB HL. However, in this experiment we used insert earpieces from the Kemp system; the psychophysical crossover in similar insert earphones exceeds 70–90 dB in frequencies below 1000 Hz and 60–70 dB in frequencies above 1000 Hz (Killion, 1984).

Liberman and Brown (1986) showed almost no efferent suppression in cats stimulated by 25 ms or less of noise stimulation; optimum durations to activate the efferents were reported to reach an asymptote between 50 and 500 ms. Within the limits of the technical difficulties to be described, our findings suggest that the human efferent system overlaps at least one part of the time-frame seen in cats.

Binaural stimulation in the forward masking paradigm predictably elicited more robust and more reliable efferent suppression of evoked otoacoustic emissions than either ipsilateral or contralateral stimulation. In absolute numerical terms, however, more suppression is seen with continuous 60 dB SPL contralateral noise stimulation when the click is at 55 dB peak Sound Pressure ($\approx$ 17 dB HL) than we see in the binaural condition in this forward masking experiment (Hood et al., 1994; Berlin et al., 1995). This observation is to be expected because of the forward masking nature of the paradigm; the continuously running noise paradigm would confound data collection during conditions of ipsilateral and binaural efferent stimulation.

5. Technical difficulties

We recognize several problems with the data presentation in this experiment. Because of constraints in the available software, the click stimuli could only be deliv-

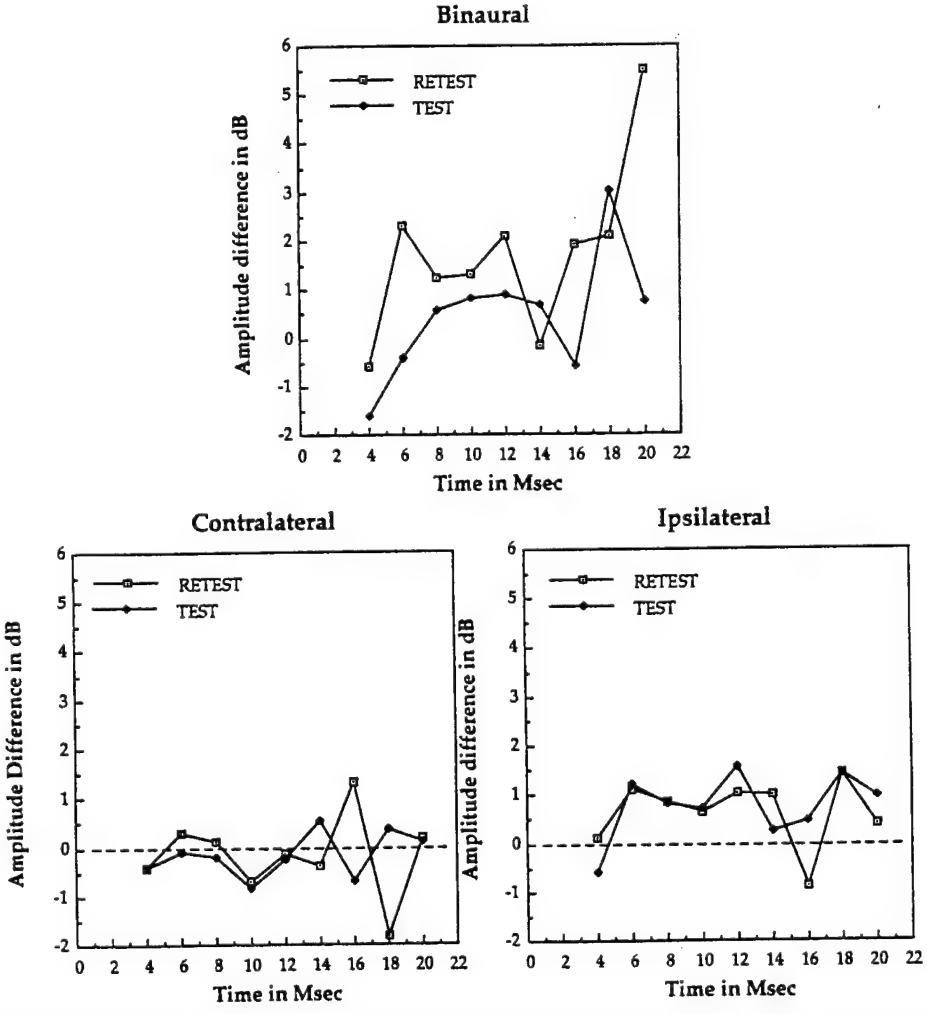


Fig. 4. One subject's test-retest reliability for the time separation of 1 ms between the end of the binaural, ipsilateral or contralateral noise.

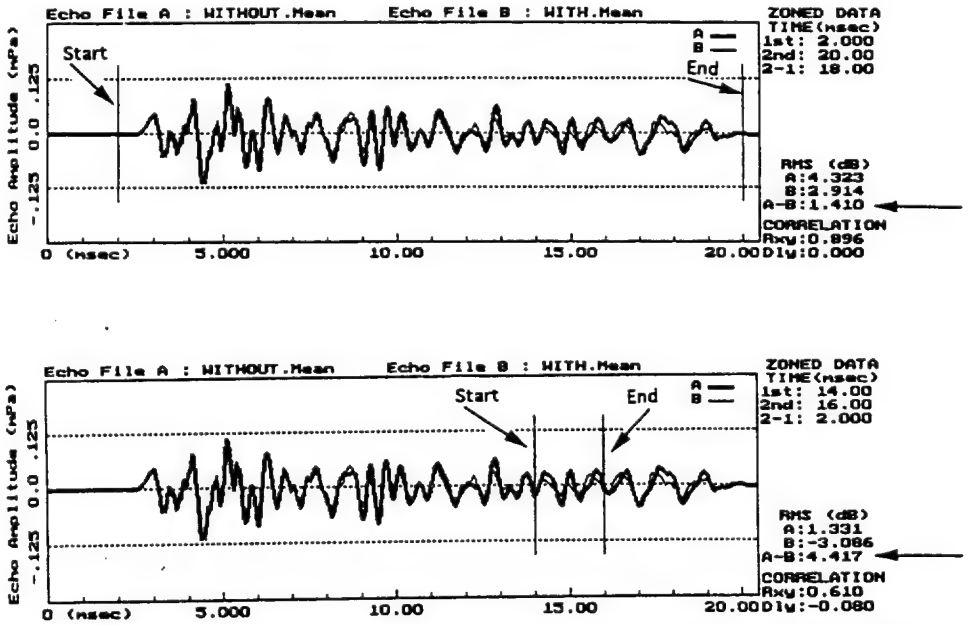
d in packets of four stimuli per stimulation unit. Thus, en we described a click train as beginning '1 ms after end of the noise,' it was only the first of the 4 clicks t was one ms away from the end of the noise. The other ee pulses were 21, 41, and 61 ms away from the end of noise respectively. Yet each of the responses to the cks is added into the average obtained by the ILO88. nilarly, in the 50 ms condition the last 3 clicks were sented 71, 91, and 111 ms after the end of the noise. us, whatever efferent effects we report here are likely to e been attenuated because three-quarters of the 600 cks used to comprise a single file were 20–60 ms later n the intended time-relationship to the end of the noise. We are indebted to G.I. Frolenkov (Frolenkov et al., 95 and Tavartkiladze et al., 1995) for pointing out to us t, in order to avoid acoustic interaction between the end the noise and the beginning of the first click, we should e allowed the noise about 5 ms to decay. This was not atal flaw in any of our data collection since only the st click in the series was within 1–5 ms of the offset of noise; all subsequent clicks were offset by at least 20

If we were to present only a single click after the end of noise, the 80 ms window averaging paradigm used by ILO88 would still include the data from the three bsequent empty 'vacated' bins as 'noise' and would enuate the apparent size of the averaged evoked emis-ons by a factor of three. [We have recently observed that 1-click experiment in this paradigm yields about the same data as a 4-click train experiment; in addition we find that there may be gender differences, laterality effects and occasionally even reverse suppression effects which have to be taken into consideration in future experiments (Berlin et al., 1995; Barham et al., 1995)].

The broad-band click and broad-band noises potentially ignore frequency specificity reported in experiments of this sort (e.g., Liberman, 1989). Thus an improved experiment would have all of the echo-evoking stimuli in the same time registration with respect to the end of the noise, would include the echoes from only a single 20.48 ms bin following the click, would take into consideration ear and gender effects, and would focus on various frequency bands, where presumably the effects might be even larger than we report here.

6. Of what value is a 3 to 6 dB effect in the auditory system?

A forward masking effect of 3–6 dB-K suppression of hair cell activity, which we saw when both ears were exposed to approximately a half-second of noise, would be even larger if it could be measured while the noise were continuously active. This work supports Liberman's prediction that 200 ms or more durations of noise would adapt outer hair cell function leading to a change in the excitation pattern of inner hair cells and single units. Liberman proposed that the presence of the noise probably changed the baseline operating characteristics of the outer hair cells



g. 5. Two averaged emissions traces (Echo File A = the mean of three trials without noise compared to Echo File B = the mean of three trials with noise) pear to have only 1.410 dB difference between them when scanned from 2 through 20 msecs (vertical bars marked **Start** and **End**) but, in fact, have 417 dB amplitude differences between them when scanned between 14 through 16 msecs after stimulation. See numerical codes to the right of each splay for clarification. Companion Table 1 shows actual data values for these traces for Time Segment in msecs, Rxy (correlation), RMS differences, and me Delay between traces in msecs.

and would adapt single units faster, in preparation for upcoming transients; if such a shift were to be applied to the sharply rising edge of a speech intelligibility curve such as the articulation index, (Pavlovic, 1994) it could conceivably improve connected speech intelligibility in a borderline noisy situation by as much as 40–60% (see for example Humes et al., 1986; Pavlovic, 1994; Hood et al., 1991). Thus, shifts in the baseline from which listening in noise takes place, or anti-maski9X phenomena as outlined by Hirsh (1948), Licklider (1948), Nieder and Nieder (1970), Kawase et al. (1993), or Kawase and Liberman (1993), might be traced to outer hair cell changes controlled by the efferent nervous system, which helps to facilitate listening in noise. We would not expect the effects of efferent function to be dramatic and outstanding in humans (cf., Scharf et al., 1994) without the conditions which mimic real-life listening conditions, including (preferably) binaural presence of long durations ($\geq$ 200 ms) of noise. However, Henson et al. (1994) suggest that cochlear reverberation may be reduced through activation of the efferents in bats. A similar reduction in cochlear reverberation, as yet unreported in humans, would serve to essentially improve the signal-to-noise ratio whenever it occurred.

7. Relationship to other forward masking work on emissions

Kevanishvili et al. (1992) and Gobsch et al. (1992) presented work studying the relationship of forward masking to emission suppression. They studied whether perceptual masking occurred at the hair cell and cochlear partition level, or at more central levels. They assumed that if masking were taking place at the pre-neural level, perceptual masking and masking of the emissions would occur at the same intensities and durations. They reported that the perceptual masking functions and masking of the emissions were quite separate, and in fact used maskers of 80 and 68 dB SPL to prove that point. Whether or not they induced middle ear muscle reflexes is debatable, but the 5 ms delay between noise offset and click onset should have vitiated most if not all of those effects. Yet these conscientious workers did not report that they saw much masking of the emissions. Our contention, after looking at their waveforms, is that there are many amplitude and phase changes in the tracings that they published, which could be easily ignored by someone looking simply at patterns but that could be quantified by summing traces from similar conditions and overlapping conditions with and without masking. Any deviations from perfect correlation might be due to either noise or phase and amplitude changes. Our analysis program is capable of quantifying such a change, where the eye is not. For example, using data that do not appear to show much difference between traces by casual inspection, we can show differences of 4.417 dB (Fig. 5) between 14 and 16 ms but only 1.4 dB when viewed globally over a 2–20 ms period after stimulation. Such minuscule visual differences in Gobsch et al. (1992) and Kevanishvili et al. (1992), could easily be overlooked by naked eye scanning unless one overlapped the traces and made a systematic point-by-point analysis. The numbers from such an analysis, taken in 2 ms steps, is provided in Table 1. The first column lists the selected time segments. The second column shows the correlation between the 'without' and 'with' noise traces in each time segment, the third column the RMS amplitude differences in dB in each time segment, and the fourth column shows any shifts in ms between the two traces. The resolution here is 40 μs per point.

Table 1
Echo R_{XY}, RMS differences and delay values for Fig. 5

Time (ms)	R_{xy} (x = A, y = B)	RMS (A–B in dB)	Delay (B to A in ms)
2.00– 4.00	0.972	0.14	0.000
4.00– 6.00	0.964	0.35	0.000
6.00– 8.00	0.950	0.99	0.000
8.00–10.00	0.867	1.96	−0.040
10.00–12.00	0.913	2.36	−0.040
12.00–14.00	0.836	3.21	−0.040
14.00–16.00	0.610	4.42	−0.080
16.00–18.00	0.771	2.48	−0.080
18.00–20.00	0.809	3.72	−0.040

One other difference must be addressed. Gobsch and Kevanishvili first collected emissions data without masking. Then they conducted their masking experiments and, at the end of the masking experiments, collected emissions again to clicks alone. They did not report any changes between the control conditions before and after the experiment. We have found that unless we alternated the conditions of testing (three conditions each of 'without' alternated with three conditions of 'with' masking) we would see a small but measurable change in the baseline of emission amplitude without any contralateral stimulation, which could have obscured our visualization of experimentally induced suppression.

8. Conclusions

We studied the suppressive effects of binaural, contralateral and ipsilateral white noise on linear TEOAEs. Binaural stimulation elicits the most suppression of otoacoustic emissions in a forward masking paradigm when the onset of the click train is 20 ms or less after the offset of a 408 ms white noise burst. Less suppression occurred to ipsilateral or contralateral stimulation, and the suppression essentially disappeared when the end of the noise was 100 ms or more away from the beginning of the click train.

owledgements

DCD Center Grant P0 1 DC-000379, Training Grants
)C-00007, Department of Defense Neuroscience Cen-
ant via N. Bazan, Kam's Fund for Hearing Research,
Cleberg Foundation, Lions' Eye Foundation and Dis-
-S Charities, NIDB BMDR-1549.

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Suppression of Otoacoustic Emissions in Normal Hearing Individuals

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INTRODUCTION

The recent discoveries of evoked otoacoustic emissions (Kemp, 1978) and of outer hair cell motility (Brownell, Bader, Bertrand, & deRibaupierre, 1985) have contributed to a significant increase in the understanding of the role of cochlear micromechanics in the processing of auditory stimuli. Otoacoustic emissions are low-level sounds that emanate from the cochlea and can be recorded from the external ear canal using sensitive low-noise microphones. They are associated with nonlinear processes present in the normal cochlea and enhanced sensitivity and tuning of the auditory system. The origin of otoacoustic emissions is ascribed to processes associated with the mechanical motion of the outer hair cells, thought to be controlled through the efferent auditory pathways via the olivocochlear system (Kemp, 1978; Kemp & Chum, 1980; Probst, Lonsbury-Martin, 1991; Norton & Widin, 1990).

There are two broad categories of otoacoustic emissions (OAEs): spontaneous and evoked (Table 3–1). Spontaneous otoacoustic emissions, or SOAEs, by definition occur spontaneously, requiring no external stim-

TABLE 3–1. Classification of otoacoustic emissions.

Category	Type	Stimulus
Spontaneous		None
Evoked	Transient	Clicks or tonebursts
	Distortion Product	Pairs of pure tones
	Stimulus Frequency	Swept pure tones

ulus to elicit a response. In contrast, evoked emissions fall into three classes, each requiring a different type of external stimulus. Transient evoked otoacoustic emissions (TEOAEs) are obtained in response to brief stimuli such as clicks or tonebursts, distortion product otoacoustic emissions (DPOAEs or DPEs) are produced by pairs of pure tones, and stimulus frequency emissions (SFOAEs) are generated by presentation of continuous tonal stimuli. Otoacoustic emissions have received extensive attention in human and animal research and several reviews are available (e.g., Glattke & Kujawa, 1991; Martin, Probst, & Lonsbury-Martin, 1990; Probst et al., 1991). Clinical applications have focused on TEOAEs and DPOAEs in the evaluation of cochlear function, screening for hearing loss in newborns, and monitoring changes in auditory function.

Emissions are not only valuable in analyzing the integrity of individual ears; they can also be used to evaluate interactions between the two ears by studying emission suppression following presentation of additional stimuli to the same, opposite, or both ears. A number of studies have described suppression of spontaneous and transient emissions in humans by contralateral acoustic stimuli (Berlin, Hood, Cecola, Jackson, & Szabo, 1993a; Collet et al., 1990; Grose, 1983; Schloth & Zwicker, 1983; Mott, Norton, Neely, & Warr, 1989; Rabinowitz & Widen, 1984; Ryan, Kemp, & Hinchcliffe, 1991; Veuillet, Collet, & Duclaux, 1991). These suppression effects observed in humans are consistent with suppression of both cochlear emissions and auditory nerve activity observed in animals (e.g. Buno, 1978; Liberman, 1989; Mountain, 1980; Puel & Rebillard, 1990).

Anatomical and physiological evidence supports the interdependent function of the two ears mediated through the efferent neural pathways which link one side of the auditory system to the other side via the medial and lateral components of the olivocochlear system (Warr & Guinan, 1978; Warr, Guinan, & White, 1986). The medial olivocochlear components terminate primarily on the outer hair cells, whereas the lateral olivocochlear components terminate mainly on primary auditory neurons at the base of the inner hair cells. Outer hair cell activity is believed to be modified through the medial efferent connections. Function of these pathways can be studied objectively and noninvasively in animals and in humans using suppression of otoacoustic emissions.

Suppression of transient evoked otoacoustic emissions has been a subject of study at Kresge Hearing Research Laboratory for several years. Research has focused on understanding the physiology of suppression of emissions, definition of the normal characteristics of suppression, parametric study of stimulus and external factors that affect suppression, the development of methodology that leads to efficient and reliable recording and analysis of suppression, and delineation of clinical applications. In this chapter, we focus on the characteristics of suppression in individuals with normal auditory function and describe the effects of stimulus and noise characteristics on suppression and the definition of suppression effects through specialized analysis techniques. This chapter is followed by a companion chapter which describes clinical patients who show no suppression of emissions and discusses ways in which suppression information can be used in the management of hearing loss.

Subjects

Subjects participating in the following studies all had normal hearing in both ears, defined as thresholds of 15 dB HL or better for the frequency range of .25 through 8 kHz. Middle ear measures (tympanograms and ipsilateral and contralateral acoustic reflex thresholds) were within normal limits and subjects had no history of neurological abnormality.

General TEOAE Recording Methods

For the studies described here, transient-evoked otoacoustic emissions were obtained using an Otodynamics ILO88 otoacoustic emissions system. Stimuli were 80-microsecond clicks which were nonlinear in our early studies, but linear in more recent studies. Nonlinear click trains present three stimuli of like phase followed by a fourth stimulus both opposite in phase and 10 dB higher in intensity. This type of stimulus paradigm is desirable in general emissions test situations to reduce stimulus artifact while maintaining a quantifiable otoacoustic emission. However, use of nonlinear click trains affects the true amplitude of the emission and thus makes absolute quantification of suppression effects difficult. Thus, the use of linear clicks (all clicks of like phase) is both desirable and possible since lower intensity stimuli are generally used in suppression studies. Suppressor (masker) stimuli are generated either externally (when contralateral noise is presented simultaneously) or internally by the ILO88

system (in a forward masking paradigm). When externally generated, noise levels are continually monitored with a probe microphone.

Three control (without contralateral noise) and three experimental (with contralateral noise) test conditions are alternated and averaged separately for each subject prior to analysis in accordance with the method suggested by Collet et al. (1990). Averages of 260 click trains of 4 clicks each are obtained for a total of 1,040 stimuli per condition, unless otherwise noted. Responses are accepted when the stimulus stability exceeds 80% and the response reproducibility exceeds 70%. Stimulus stability represents a comparison of stimulus level recorded in the ear canal at the beginning of the test to the level of the stimulus monitored throughout the test acquisition period. Response reproducibility represents the correlation of averages of half of the sweeps which are stored in one computer buffer and the other half of the sweeps stored in another memory buffer. These two averages are acquired by interleaving sweeps between two computer memories during the acquisition of an emission. A typical evoked otoacoustic emission obtained from a female subject with normal hearing using the ILO88 System is shown in Figure 3–1. In this case, the overall amplitude of the emission is 12.9 dB and the response reproducibility is 98%. The stimuli were linear clicks which were monitored at

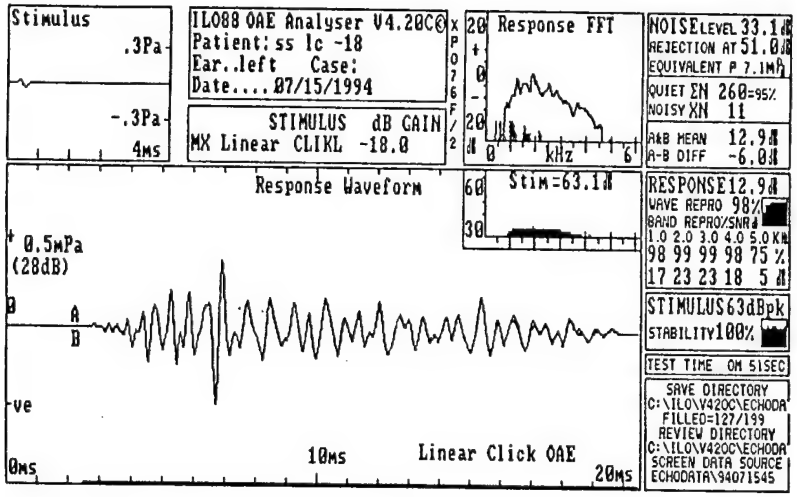

Figure 3–1. Transient Evoked Otoacoustic Emissions (TEOAEs) obtained from a normal subject using linear clicks.

an intensity of 63 dB peak sound pressure in the ear canal, and the stimulus stability during the test was 100%.

Early Studies

In our early studies of suppression, transient otoacoustic emissions were obtained using nonlinear clicks at 80 dB peak SP (sound pressure). The emissions were suppressed by either pure tones (at octave intervals from 250 to 4kHz), narrow band noises (centered at octave intervals from 250 to 4kHz), or broad band noise presented simultaneously to the contralateral ear. Results indicated greater suppression with increasing masker levels, greater effects with narrow-band noises than with pure tones, and greater suppression effects for lower frequency than higher frequency maskers. The details of these studies were reported in Berlin et al. (1993a).

In all of our studies, we reported suppression effects as dB differences in emission amplitude between conditions with and without noise rather than as equivalent dB, as Collet and colleagues have used (Collet et al., 1990; Veuillet et al., 1991). Although the differences observed in overall emission amplitude between conditions without and with noise appear small (on the order of 1 or 2 dB), they are quite consistent. We also observed that suppression effects are greater in certain time periods, which led us to the development of a method to analyze suppression of emissions in greater detail.

Development of Data Analysis Techniques

As we sought to refine our understanding of suppression, one of the authors (HW) developed an analysis program to quantify changes in transient evoked otoacoustic emissions obtained under varying test conditions (Wen, Berlin, Hood, Jackson, Hurley, 1993). The Kresge EchoMaster Program (current version 3.1) is compatible with the Otodynamics ILO88 file structure and allows detailed comparisons of root-mean-square (RMS) amplitude, cross correlations, and time delays across the entire time window or in selectable time periods. Frequency domain data can also be obtained using fast Fourier transforms (FFT) and selectable windowing functions to quantify emission spectra. The EchoMaster program provides comparisons of (1) two individual emissions, (2) the means of two groups of emissions with up to 60 emissions in each group, and (3) an individual emission with its estimated background noise. An example of the data obtained from the EchoMaster analysis is shown in Figure 3–2.

Prior to addition of data, like conditions (i.e., the three conditions without noise and the three conditions with noise) are reviewed for data

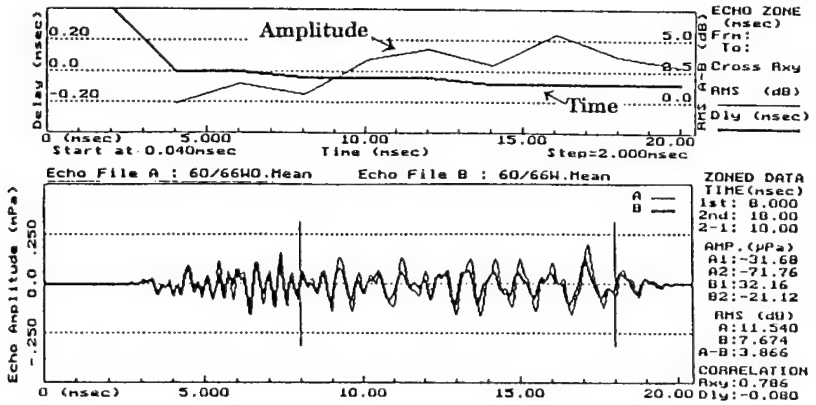

Figure 3–2. Analysis method for quantification of suppression using the Kresge EchoMaster program. Emissions obtained without (thin line) and with (thick line) are shown in the bottom portion. The top portion shows amplitude and time differences between the without and with conditions in 2-ms intervals.

consistency. Mean data are obtained for emissions from like conditions (i.e., without or with noise) only within subjects, and each subject's emissions are compared only with his or her own emissions. Once emission suppression is quantified for individual subjects through the EchoMaster analysis, we then can compare data across subjects.

CHARACTERISTICS OF TEOAE SUPPRESSION

What Are the Characteristics of Suppression in Normal Individuals?

Suppression of TEOAEs is characterized as a reduction in amplitude and/or a time change or phase shift (e.g., Berlin et al., 1993a; Collet et al., 1990; Ryan et al., 1991; Veuillet et al., 1991). Subjects vary in the amount of suppression, although in our experience all normal subjects show amplitude decreases with noise in some time periods. Some subjects demonstrate suppression of as much as 5 to 7 dB. As the intensity of the contralateral noise increases, the amount of suppression increases and time periods involved tend to broaden.

Suppression also is demonstrated as a change in the time of occurrence of peaks or zero crossings in the emissions, particularly at later time periods. Introduction of contralateral noise causes a decrease in the latency of peaks relative to the stimulus, or a "negative" time delay. This can be seen in the lower tracing of Figure 3–2 where the peaks in the suppressed condition are slightly earlier, particularly in later time periods. As the intensity of the contralateral noise increases, earlier time periods tend to be involved as well for both amplitude and time differences (Hood, Hurley, Wen, Berlin, & Jackson, 1993).

Test-retest reliability analyses have been incorporated into many of our studies of suppression. Results indicate good repeatability of suppression effects for both amplitude and time within subjects as well as consistent group effects (e.g., Hood et al., 1993).

Is Suppression Greater in Certain Time Periods?

A consistent observation throughout our studies is the occurrence of maximal suppression in the 8- to 18-ms time period which is consistent with other reports (e.g., Collet et al., 1990). Analysis by 2 ms intervals, as shown in Figure 3–3, indicates that suppression varies across time and that the greatest amplitude suppression and time delay changes occur in the time period between 8 and 18 ms. In this figure, data obtained from the EchoMaster analysis of a series of normal subjects have been combined to examine mean suppression effects on amplitude (left panel) and time (right panel). Very little suppression is observed from 2 to 8 ms in either the amplitude or time graphs. Effects across this group of subjects increase with time and are greatest in the 8- to 18-ms regions. Boxes outline the time window that is used to quantify suppression effects in our subsequent studies.

The "Echo in dB" value from the ILO88 system describes the characteristics of an entire emission as a single value. Because suppression effects are greater in some regions than in others, a single value cannot adequately represent the maximal effects or define time regions containing the greatest amount of suppression. In fact, an overall aggregate number will underestimate the suppression effect in most subjects. Thus, we recommend point-by-point analysis of suppression at 1-, 2-, or 3-ms time intervals to identify time regions of maximum effect and more precise quantification of suppression.

An example of a subject with minimal suppression when analyzed with the overall ILO88 number is shown in Figure 3–4. Although this single number might suggest the lack of a suppression effect, this subject shows substantial suppression between 10 and 12 ms. In addition, some subjects show greater amplitude changes while other subjects may show greater temporal changes.

Do Intensity Levels of the Click and Noise Affect Suppression?

We reasoned that because otoacoustic emissions are associated with cochlear processes occurring in response to low-intensity stimuli, it is

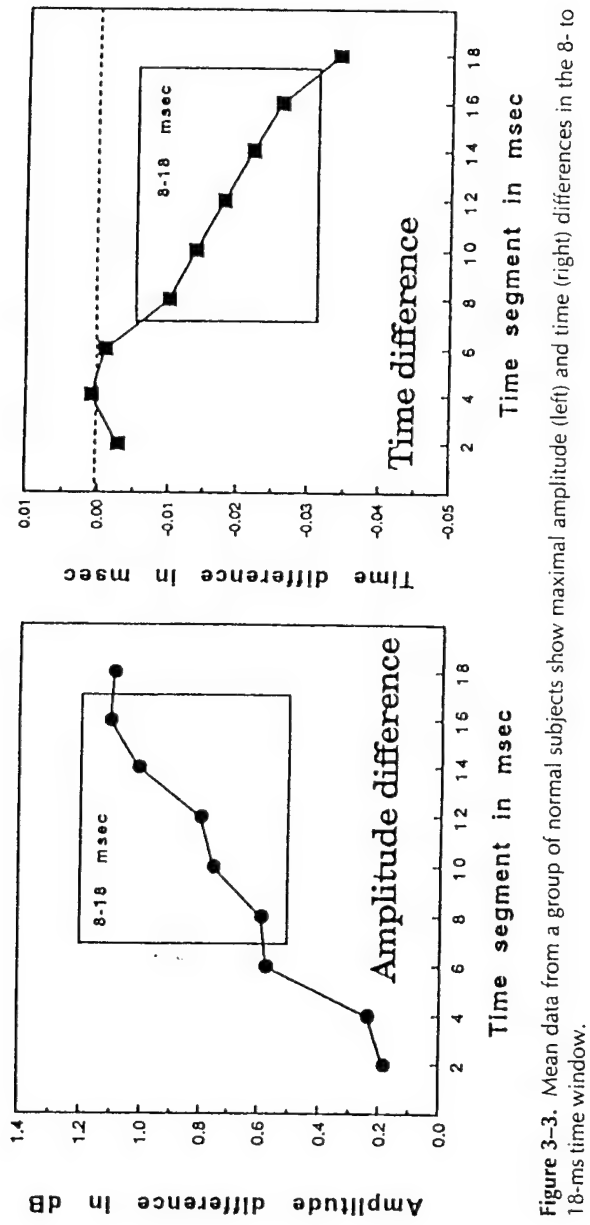

Figure 3–3. Mean data from a group of normal subjects show maximal amplitude (left) and time (right) differences in the 8- to 18-ms time window.

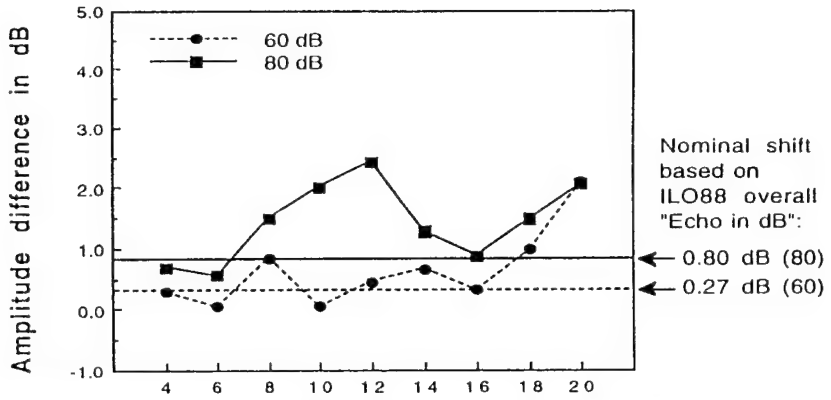

Figure 3–4. Some normal subjects who appear to have little contralateral suppression calculated in overall dB by the ILO88 system show clear amplitude changes in isolated time periods. Shown are data for one subject who shows less than 1 dB of suppression in the aggregate echo but more than 2.4 dB suppression in the 10–12 ms time period.

possible that fainter click and noise levels may be optimal for observing suppression effects. In one study, we varied the intensity of both linear clicks and contralaterally presented continuous white noise to determine the optimal click and noise levels that would yield the greatest contralateral suppression effect (Hood, Berlin, Hurley, Cecola, & Bell, 1994).

Comparison of suppression for linear clicks of peak sound pressures from 50 to 70 dB and contralateral white noise from 10 dB below to 10 dB above the click peak SP showed that the amount of suppression is dependent on both the level of the suppressor noise and the level of the stimulus eliciting the emission. Suppression increased systematically as the level of the noise was increased. Maximum suppression was reached with clicks of 55 dB peak SP and above (Figure 3–5). The observation of a decrease in suppression for 65 dB peak SP clicks suggests the possibility of different "low" and "high" level processes affecting suppression, depending on the stimulus and noise intensities.

The occurrence of greater suppression for some lower rather than higher intensity stimuli described here also reduces concern about contamination of suppression by either acoustic crosstalk or acoustic reflexes, both of which may contribute at high intensities but could not have a greater effect at lower than higher intensities.

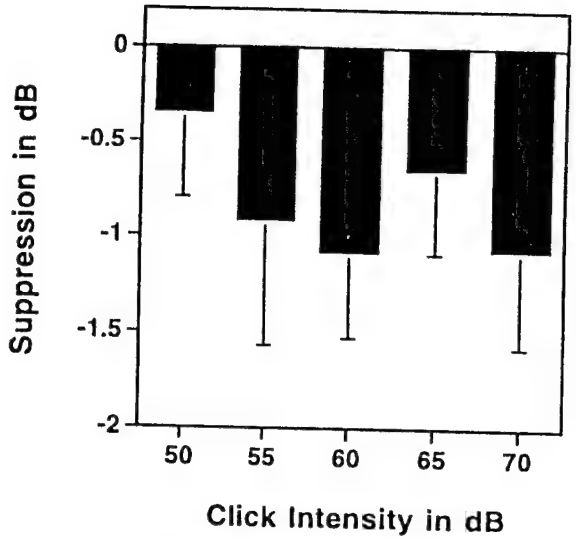

Figure 3–5. Effects of stimulus and suppressor intensity on suppression. The amount of suppression increases with increasing suppressor intensity, but is greater for some lower intensity stimuli.

BINAURAL, IPSILATERAL, AND CONTRALATERAL SUPPRESSION

The olivocochlear system can alter outer hair cell activity when activated by contralateral, ipsilateral, or bilateral stimuli. Although most experiments on suppression of otoacoustic emissions have involved contralateral simultaneous stimuli, Berlin, Hood, Hurley, Wen, and Kemp (1995) extended TEOAE suppression studies to the presentation of ipsilateral and bilateral suppressors as well as contralateral suppressors. In order to accomplish this, it was necessary to develop a forward masking paradigm which allowed temporal separation of the suppressor and the emission evoking click. This was accomplished through the assistance of Dr. David Kemp, who provided the software necessary to complete a forward masking paradigm using the Otodynamics ILO88 system.

Forward Masking Paradigm

In a forward masking paradigm, the masker precedes the test stimulus in time. By separating the masker and stimulus in time, acoustic interaction

of the two signals is minimized. Even though a masker precedes the test stimulus, psychophysical studies have shown that a masking effect persists for a short time after the cessation of the masker.

To study the effects of binaural, ipsilateral, and contralateral noise on suppression we collected TEOAEs in a forward masking paradigm using linear clicks at 65 dB peak SP and a 65 dB SPL white noise masker that was 400 ms in duration (Figure 3–6). Time separations between the offset of the noise and the onset of the first click of a four-click series were varied from 1 to 200 ms. Because the ILO88 presents stimuli in groups of four, the three clicks following the initial stimulus were each separated by an additional 20 ms. Thus, only the first click in the series was masked according to these delay times, a limitation that we are currently correcting with another paradigm which allows presentation of a single stimulus following the masker.

Do Ipsilateral and Bilateral Noise Stimuli Yield Results Similar to Contralateral Noise?

Binaurally presented noise resulted in significantly greater suppression than ipsilateral or contralateral noise (Figure 3–7) and contralateral noise was the least effective suppressor (Berlin et al., 1995). Consistent with previous studies, the greatest suppression occurred between 8 and 18 ms following stimulus onset. Mean maximal suppression effects were on the order of 3.0 to 3.5 dB for binaural noise, 1.5 to 2.0 dB for ipsilateral noise, and 1.0 to 1.5 dB for contralateral noise.

Does the Time Separation Between the Noise Offset and the Click Affect Suppression?

The literature from behavioral studies of forward masking indicates that the effectiveness of a masker decreases as the time separation between

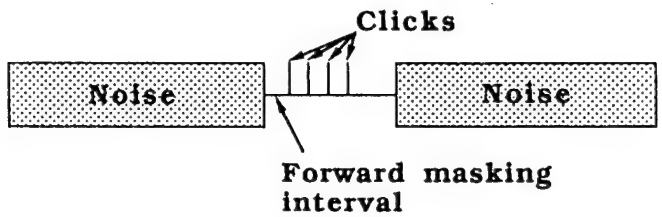

Figure 3–6. Diagram of a forward masking paradigm used to evaluate suppression of emissions.

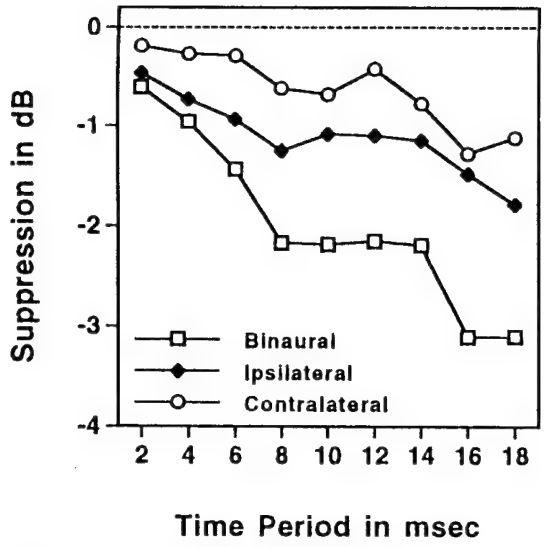

Figure 3–7. Comparison of suppression with ipsilateral, contralateral, and binaural noise. Binaural stimulation is more effective than either ipsilateral or contralateral noise.

the offset of the masker noise and the onset of the stimulus increases. Consistent with behavioral studies, the amount of emission suppression decreased systematically with increasing time separations between the masker and the click train (Berlin et al., 1995). One ms separation yielded the most suppression, and little or no suppression was observed when the noise and clicks were separated by 100 to 200 ms. It should be noted that these suppression effects may underestimate the actual suppression due to the additional time delay between the noise and the second, third, and fourth clicks in the train. When the time separation between a masker noise and a click is less than 10 ms, it is also possible that the noise itself will produce an emission that will contaminate the target response (Tavartkiladze et al., 1995). Thus, a time separation of 10 ms between the noise offset and stimulus onset appears to yield a suppression effect with minimized contamination.

Does Noise Duration Affect Suppression?

Liberman and Brown (1986) first showed that stimulation of 50 to 500 ms is required to obtain responses from olivocochlear neurons. For this reason, we investigated the effects of noise duration in a forward-masking paradigm on TEOAE suppression (Hood, Berlin, Wakefield, & Hurley, 1995). We compared broad-band noise durations from 80 to 640 ms (at 65 dB SPL) presented prior to the onset of 65 dB peak SP linear clicks. Increases in noise duration yielded progressively greater suppression through 400 ms with no significant increase in suppression for noise durations from 400 to 640 ms. These data extend to humans the observations made by Liberman and Brown (1986) that a minimum stimulus duration is necessary for activation of the efferent system.

Potential Problems and Other Considerations

Several factors have the potential to contribute to a reduction in emissions when noise is introduced. These include activation of the middle ear muscle reflexes and acoustic crosstalk or crossover of sound through the head. However, several factors reduce the possibility that either of these factors plays a major role in the observed suppression effects. Our data showing greater suppression effects for lower intensity stimuli minimize the potential role of the middle ear muscle reflex and crossover, as effects would be expected to increase with intensity if they represented either of these phenomena. Acoustic crossover is also unlikely because we used insert earphones with 60–90 dB interaural attenuation (Killion, Wilbur, & Gudmundsen, 1985). In addition, both our group and Collet and his colleagues have observed suppression in patients who lack stapedial muscle function due either to Bell's Palsy or stapedial tendon section during stapedectomy.

It is important to monitor click and noise levels during evaluation because these levels vary greatly across ears. Consistent with Collet and his colleagues, we also recommend use of linear rather than nonlinear clicks and presentation levels in the range of 55 to 60 dB peak SP in order to avoid the potential contaminants discussed.

Clinical Application

Based on our studies, we measure suppression of transient evoked otoacoustic emissions clinically using 55 or 60 dB peak SP linear clicks and noise levels of 55 to 65 dB SPL. We interleave three "without noise" and three "with noise" conditions, analyze results using the Kresge EchoMaster software, and focus on the 8- to 18-ms time period. In patients of interest, we present bilateral, ipsilateral, and contralateral noise in a forward masking paradigm. We believe that suppression of otoacoustic emissions provides insight into function of the efferent system and the interactions between the afferent and efferent pathways which allow us to distinguish

central from peripheral hearing losses and manage both types of hearing disorders more accurately (e.g., Berlin et al., 1993b; Williams, Brooke, & Prasher, 1994).

SUMMARY

The series of studies summarized in this chapter provide information about some of the characteristics of suppression of transient evoked otoacoustic emissions in adults with normal hearing. These characteristics of suppression can be summarized as follows:

1. Suppression is characterized by amplitude decreases as well as time shifts of emission peaks.
2. Suppression is greatest in the 8- to 18-ms time period.
3. Some normal subjects who appear to have little or no suppression when a single value is calculated to represent the entire 20.48 ms period show clear suppression in the 8- to 18-ms range.
4. As the intensity of the suppressor noise increases, suppression amplitude and time differences increase.
5. Suppression is greater for lower intensity stimuli than for higher intensity stimuli.
6. Suppression is greater for binaural noise than for ipsilateral or contralateral noise.
7. Suppression is greatest for time separations of less than 10 ms and for noise durations greater than 400 ms.
8. Test and retest comparisons show that suppression effects are repeatable.
9. Suppression of otoacoustic emissions is useful clinically in evaluating and managing patients with central and peripheral hearing losses.

Acknowledgment: This work was supported by NIH NIDCD P01-DC00379, DOD-DAMD1783V3013, Kam's Fund for Hearing Research, the Kleberg Foundation, and the Louisiana Lions Eye Foundation. We extend appreciation to David T. Kemp, Ph.D. for providing the forward masking software. We also acknowledge the following individuals for their participation in data collection: Betsey Bell, M.C.D., R. Patrick Cecola, M.D., Dena Jackson, M.A.T., Laura Wakefield, M.A.

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A Singular Audiology Text
Jeffrey L. Danhauer, Ph.D.
Audiology Editor

Neurotransmission and Hearing Loss

Basic Science, Diagnosis, and Management

Proceedings of the Second Annual
Kresge-Mirmelstein Symposium
in Honor of Robert Wenthold, Ph.D.
Held in New Orleans, September 29, 1995

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SINGULAR PUBLISHING GROUP, INC.
SAN DIEGO · LONDON

7

Autoimmune Inner Ear Disease: Basic Science and Audiological Issues

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INTRODUCTION

In the lay literature hearing losses are divided into convenient if not valid artificial compartments: **conductive loss** affecting the outer and middle ear and **nerve loss** affecting the inner ear (e.g. Consumer's Union, 1992; many commercial messages from Beltone and Miracle Ear). Physicians, speech-language pathologists, nurses, and audiologists occasionally may resort to these classifications in counseling with patients in order to avoid "complicated explanations." A common consequence is that many patients and their families conclude that the patient has a "nerve loss" and cannot be helped either by medicine or hearing aids since there is a common misperception in the lay public that "nerve fibers cannot regrow."

This semantic trap surrounding **"nerve deafness"** also leads to failure on the part of gatekeeping physicians to refer patients for audiological or otologic service because "nothing can be done anyway" for patients with sudden or exacerbated sensorineural hearing losses. We discuss here the history and current status of a type of inner ear disease that is medically diagnosable and treatable, for which hearing aids are also currently available, and add to this mixture the discussion of a type of truly neural or at least inner hair cell hearing loss that is self-limiting and from which some patients seem to recover in part if not completely.

THE IMMUNE SYSTEM

The immune system defends the body against what it identifies as "foreign invaders"; however, it sometimes makes errors and misidentifies indigenous tissues as "foreign." It is this error that generates an autoimmune disease.

In a normal immune response an antigen (a substance such as a virus, bacterium, fungus, parasite, foreign body, or cells from another organism capable of triggering an immune response) is first attacked by monocytes, but whatever portion of the invader remains permanently changes the body's subsequent response so that future reactions are different from the initial reaction.

Lymphocytes are white blood cells with primary immune responsibility; they come in two forms, T cells and B cells. T cells have the unique property of binding to antigens and forming lymphokines, which attack and destroy the antigens, while the B cells generate immunoglobulins to fight the antigens with special vigor after the first infection. An excellent example is the fetus' nonreactive response to Rh incompatibility only if it is the mother's first pregnancy; if it is part of a subsequent pregnancy, the infant may develop a potentially fatal autoimmune response.

There are many theories suggesting that autoimmune diseases are a concomitant of age, viruses, drug usage, or have genetic underpinnings (Berger, Hillman, Tabak & Vollrath, 1991; Playfair & Lydyard, 1995; Ruckenstein & Harrison, 1991; Veldman, 1989), but the exact mechanisms are poorly understood.

HISTORY AND BACKGROUND

Autoimmune diseases have been categorized as either organ-specific or systemic (Schleuning & Andersen, 1993; Harris & Ryan, 1995), and there are many systemic autoimmune diseases which can cause hearing loss. These include among others lupus erythematosis, Wegener's granulomatosis, rheumatoid arthritis, Cogan's syndrome, relapsing polychondritis, and diabetes and syphilis, which in themselves can be considered forms of systemic autoimmune and/or inflammatory diseases. Thus in patients with fluctuant or progressive hearing losses, contributions from syphilis, diabetes, or thyroid disease must also be ruled out.

The inner ear and brain were once thought to be inaccessible to systemic autoimmune diseases (Harris & Ryan, 1995), but the inner ear contains lymphocytes, which "guard the ear" against infection, suggesting that certain infectious processes might in fact attack the ear directly. Immunosuppressant drugs were first used by Schiff and Brown (1974) for the treatment of sudden hearing loss caused by an autoimmune disease, but it was McCabe (1979) who first showed that some inner ear losses were reversible by the judicious use of steroids and other anti-inflammatory or immunosuppressant agents. In following 66 patients with rapidly progressive sensorineural loss McCabe and McCormick (1989) reported that cyclophosphamides and/or prednisolone were effective in limiting loss of and/or improving auditory function. To date, it is still common otologic practice to treat diagnostically; that is to say, if the patient were to respond to steroids, the etiology would be presumed to be autoimmune or inflammatory.

Hughes, Barnes, Calabrese, Kinney, and Nalepa (1988) suggest that the prototypical patient is 20–50 years of age, often female, and has bilateral asymmetrical rapidly progressive inner ear loss of no particular audiometric configuration. Fifty percent of his patients reported vertigo, and 25–35% presented with other systemic autoimmune disease, such as rheumatoid arthritis or lupus erythematosis. He recommends specific tests for the presence of autoimmune disease and, if clinically appropriate, offers immunosuppressant therapy in the form of steroids or cytotoxic drugs. One audiologic characteristic on which Hughes focuses strongly is that he believes, in autoimmune compromised patients, speech discrimination scores are poorer than one would expect from the audiogram.

MAKING THE DIAGNOSIS

The laboratory tests for inner ear autoimmune disease include the Western Blot immunoassay and the Lymphocyte Transformation Test (Hughes, Moscicki, Barna, & San Martin, 1994). The Western Blot assay has a sensitivity of 88% and a specificity of 71%. In a low-risk population the predictive value of the test is less than 40%, but in high-risk populations, the predictive value is well above 90%. In addition, if the Western Blot test is positive, it has a predictive value of 80%, but only a 30% predictive value if it is negative. The Lymphocyte Transformation Test has a sensitivity of 50–80% but a specificity of 93%; in a high-risk population its predictive value is about 56%.

Harris and Ryan (1995) report that out of 279 patients with rapidly progressive sensorineural loss, only 32% (90) were positive by the Western Blot test in the 65–70 k-dalton range. Thus, the test is highly sensitive if used in populations at high risk, but not if used in the general population.

Mogi, Lim, and Watanabe (1982) have shown that immunoglobulins exist in the perilymph of the inner ear and derive from a filtrate of perilymphatic blood vessel, since they believed but were not sure at the time that the inner ear's perilymphatic system was separate from that of the CSF. Harris (1984) later supported the notion that the cochlea's perilymphatic system is separate from that of the cerebrospinal fluid and offered convincing evidence that the inner ear produces its own antibodies.

Arnold, Pfaltz, and Alternott (1985) showed that immunoglobulins (IGa and IGg) can be found in the lumen of the endolymphatic sac and epithelial cells, supporting the notion that the inner ear has its own anti-

body producing mechanism. They also reported antibodies against healthy inner ear tissues in 15 of 21 patients with progressive or fluctuant sensory hearing loss. In patients with Cogan's syndrome they also found antibodies against corneal tissues.

There is some evidence that secondary rather than primary immune disease leads to hearing loss. Woolf and Harris (1986) showed that guinea pigs developed serum and anti-KLH titers but did not show much cochlear histopathology or auditory loss as determined by electrophysiological methods. However, after about 2 to 4 weeks, when the same animals received an inner ear challenge, they developed marked autoimmune responses that they had not shown originally.

These data suggest that there is an organ-specific rather than simply a body-wide autoimmune disease which must be described and treated. Harris and Ryan (1995) showed that 19 of 54 patients with presumed inner ear autoimmune disease showed the same 62 to 68 k-dalton antigen on Western Blot analysis that was found in the immune-reactive guinea pigs discussed above. Gel electrophoresis showed that a patient who responded to steroids had exactly the same autoantibodies as the guinea pig and reacted to antigens with the same molecular and electrical properties.

There are even proposed animal models of autoimmune inner ear disease. Wong et al. (1992) and Trune et al. (1994) have used the Palmerston North mouse which shows sclerotic lesions and bony overgrowth similar to that seen in humans with autoimmune hearing loss.

Audiologically, the patients show many of the signs of cochlear hearing loss (middle ear muscle reflexes present, no air bone gaps, absent otoacoustic emissions), but also some signs that this hearing loss is not due entirely to outer hair cell loss or destruction. Along with rapid changes in the pure tone audiogram, there is sometimes return of function after administration of steroids. The return of hearing after steroid administration mitigates strongly against a physical loss of structures. Secondly, ABRs are not always synchronous and robust, suggesting that antibodies may be forming at the base of the inner hair cells or at the spiral ganglia, or elsewhere. Thirdly, speech discrimination at 50 dB HL does not always match the Articulation Index prediction.

SAMPLE CASES OF AUTOIMMUNE DISEASE TREATED WITH STEROIDS

Case SD-1

We first saw this young man when he was 9 years old, at which time he presented with an anacusic right ear and a mild-to-moderate fluctuant loss in his left ear. His speech was good, but he showed language and educational delays proportionate to his inability to eavesdrop on conversations and thus improve his vocabulary, idiomatic usage, and information store. Table 7–1 chronicles his pure tone audiograms, Speech Reception Thresholds (SRTs), and speech discrimination scores at a constant Most Comfortable Loudness Level (MCL) over a period of 6 years.

Thirty-two months after his initial visit, he complained to his mother that he could no longer understand speech well, and the clinical tests confirmed a significant loss of speech comprehension in his good ear. He was immediately placed on 40 mg of prednisone per day for 1 week and his audiologic data returned to baseline. Fifty-two months after his first visit he again complained of loss of speech discrimination and was again given a week-long regimen of steroids, at which point his reliable auditory function returned. His WBA (Western Blot Assay) was negative.

We follow him regularly and have fit his left ear with a wide dynamic range programmable device with a number of programs, one of which is designed to help him should he have a sudden exacerbation of hearing thresholds.

Table 7–1. Serial audiometric thresholds in dB HL for the left ear which reflect changes for Case SD-1.

Date	.25k	.5k	1k	2k	4k	6k	8k	SDS	Comments
Initial visit	50	50	40	20	20	45	50	100%	CT scan & ENG: Interpreted as normal
1 mos	45	45	30	15	20	35	45	100%	Antigen nonspecific lab. results—negative
5 mos	55	55	50	15	15	20	55	92%	
9 mos	35	50	35	0	5	10	45	100%	
15 mos	40	45	40	0	10	15	50	100%	
25 mos	45	55	55	10	15	35	40	100%	
32 mos	40	60	55	65	55	50	55	72%	Prednisone 40 mg
32 mos, 1wk	35	50	50	20	30	30	45	92%	
37 mos	45	55	60	20	30	45	45	92%	
43 mos	45	60	65	30	40	50	55	100%	
49 mos	40	55	45	20	20	35	45	96%	
52 mos	55	60	55	45	30	40	50	80%	Prednisone 40 mg
52 mos, 1wk	40	45	50	20	25	35	45	100%	
55 mos	40	50	60	25	20	30	50	100%	WBA negative
61 mos	40	55	55	20	20	35	55	96%	
63 mos	45	55	50	15	20	45	50	92%	

Case KC-1

This 62-year-old physician was seen 2 days after he reported bilateral ear fullness and loss of speech comprehension in his left ear. He reported no vertigo or tinnitus at that time. Table 7–2 summarizes his basic audiologic data for the left ear; his right ear showed no changes. The unilateral nature of the loss further supports an ear-specific hypothesis for autoimmunity, rather than a system-wide illness.

At the first visit his sedimentation rate studies were elevated, consistent with some form of inflammatory process, and the patient was put on 60 mg of prednisone for 5 days.His hearing thresholds improved dramatically.

He reported a recurrence 3 months later and he was again administered the same dose of prednisone and again he showed about a 35 dB improvement in the frequencies below 2000 Hz.

This time the WBA was positive and the patient was maintained on a regimen of steroids until 21 months postonset. At that time a diagnosis of Ménière's disease was made elsewhere. Also an endolymphatic sac decompression with shunt insertion was performed; at surgery the sac was described as dry and normal in size. The patient remains on immunosuppressants.

SUMMARY OF AUDIOLOGICAL MESSAGE

Any rapid decline in sensitivity or discrimination in a 3- to 6- month period should raise a suspicion of autoimmune hearing loss. Programmable wide dynamic range compression hearing aids can often be used to follow variations and fluctuations in sensitivity and loudness growth curves. However, in the next section we will discuss a form of hearing dysfunction that is self-reversing and may be related to another form of antibody reaction at the neural level in the inner ear in response to hyperbilirubinemia.

Table 7–2. Serial audiometric thresholds in dB HL for left ear that reflect fluctuations with changes in medication for Case KC-1.

Date	.25k	.5k	1k	2k	4k	8k	SDS	Comments
Onset	80	85	65	50	60	80	80%	Elevated ESR, 60 mg Prednisone
1 mo	35	25	30	30	40	65	100%	CT normal, 10 mg Prednisone
3 mos	60	60	55	40	50	70	100%	60 mg Prednisone
4 mos	40	25	20	30	45	70	100%	WBA positive, 30 mg Prednisone
9 mos	60	45	35	35	45	75	72%	20 mg Prednisone
11 mos	50	30	35	30	40	75	92%	60 mg Prednisone
17 mos	45	30	25	25	40	70	92%	60 mg Prednisone
21 mos	45	30	25	25	40	80	90%	10 mg Prednisone
22 mos	65	60	35	35	60	90	88%	60 mg Prednisone; Pre-op
32 mos	50	30	30	25	50	75	100%	10 mg Prednisone

HYPERBILIRUBINEMIA SOMETIMES CAUSES A FORM OF SELF-REVERSING INNER EAR LOSS

Although autoimmune diseases seem to cause self-reversing or self-limiting hair cell malfunctions, hyperbilirubinemia is associated with reversible desynchronization of the Auditory Brainstem Response (ABR), which can be mistaken for severe peripheral sensory deafness (Berlin, 1996; Stein, Tremblay, Pasternack, Benerice, & Lindermann, 1996) unless cross-checked with an otoacoustic emissions test. Below we show how five patients were misdiagnosed as being peripherally deaf and recommended for power hearing aids, until we added an otoacoustic emissions test to their diagnostic battery and found that they had normal functioning outer hair cells. All five children are now showing signs of developing auditory function without hearing aids and with normal comprehension as their ABRs develop more synchrony. Some other children with similar ABRs and emission patterns still have not developed audition. We have no idea why.

Figure 7–1 shows ABRs misinterpreted as consistent with severe-to-profound deafness on one of these five children. All of the other children showed virtually identical test results. Note the inverting cochlear microphonic and later "responses" which could have easily been misinterpreted as neural synchrony if inverted polarity stimuli had not revealed inverted responses. Figure 7–2 shows a normal evoked otoacoustic emission acquired at the same time the ABR was recorded. The paradoxical coexistence of these two findings led to further study by transtympanic electrocochleography, which showed the same inversion in polarity without a latency change with intensity drops.

Since these children now seem to have emerging auditory abilities, and since their dysfunctions are coincident with hyperbilirubinemia, we are postulating that they have some form of reversible autoimmune or transmitter-blocking condition related to kernicteric deposits on or around primary auditory structures.

Acknowledgment: We thank the NIDCD, Kam's Fund for Hearing Research, Singular Publishing Group, and The Mirmelstein Family, NDIB-BMDR 1549, and the supporters of Kam's Fund for Hearing Research.

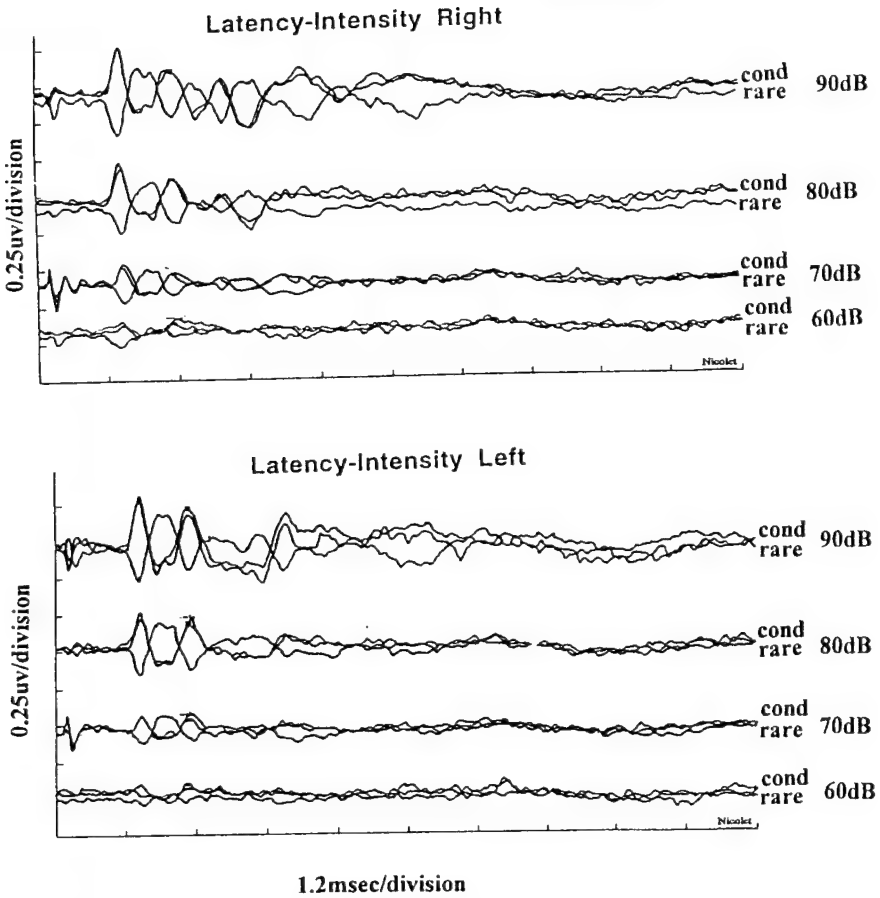

Figure 7–1. Pseudo-ABRs for right and left ears simulated by a long-ringing cochlear potential, which is identified when the polarity of the stimulus is reversed. Note how there is *no* latency-intensity function and all waves remain at the same latency even though intensity is reduced from 90 dB down to 60 dB Hln.

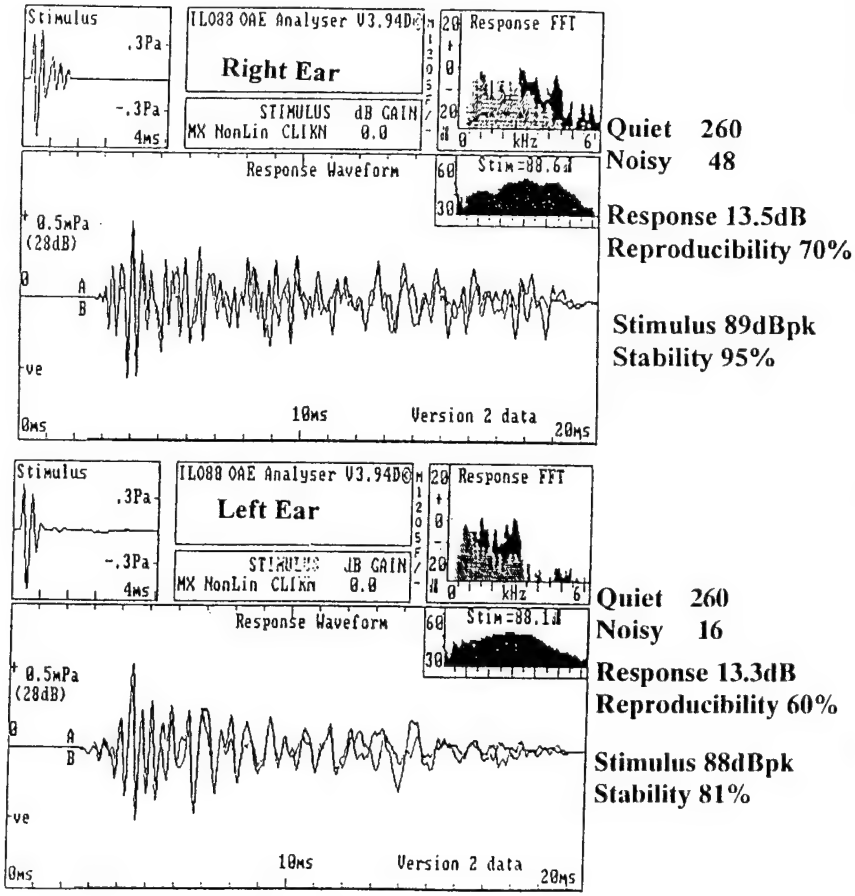

Figure 7–2. Normal evoked otoacoustic emissions from right and left ears of same sample patient.

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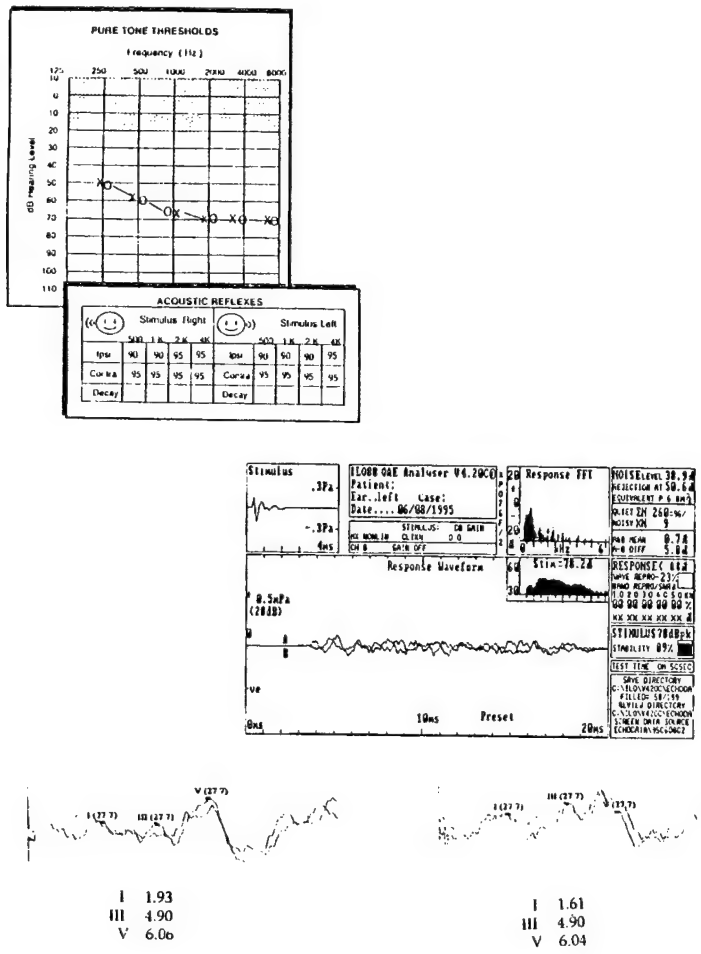


Figure 6–1. Audiogram emissions and ABR from a person with a fittable cochlear hearing loss.

ing is indicated by well-formed ABR responses with good morphology and normal absolute and interpeak latencies to high intensity, 85- to 95 dB HLn, stimuli. These are good candidates for hearing aids, and are likely to have good results, providing there are no inflammatory or autoimmune processes which disrupt speech coding.

Inner Hair Cell and Spiral Ganglion Loss

Patients who have no emissions and no ABR to air-conducted clicks are likely to have suffered some inner hair cell and spiral ganglion loss. If they are being considered for cochlear implants, electric ABRs should reveal synchronous neural discharge if properly administered and interpreted, thus, with somewhat circular reasoning, confirming their candidacy for cochlear implantation. However, many patients are successful implant candidates despite poor electrical ABR tests.

Primary Neuropathies

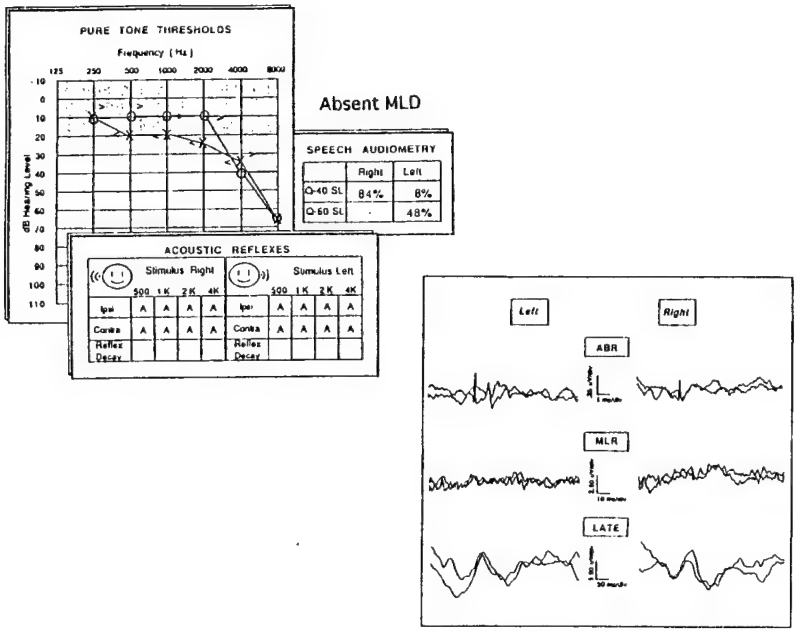
Finally, there is a small group of patients who have no ABR but have normal hair cell emissions. Some of these patients show little or no hearing loss by pure tone audiometry (Figure 6–2), others show very poor audiograms (Figure 6–3), still others have thresholds that fall somewhere in between (Figure 6–4). All show: (a) absent middle ear muscle reflexes, (b) absent MLDs, (c) very large otoacoustic emissions, and (d) virtually no efferent effects of contralateral ipsilateral or binaural noise on their robust click-evoked otoacoustic emissions (see sample in Figure 6–5 and explanation later; Berlin et al. 1994; Sininger, Hood, Starr, Berlin & Picton, 1995; Starr et al., 1991). Hood, Berlin, Hurley, and Wen have shown how we quantify efferent suppression in Chapter 3.

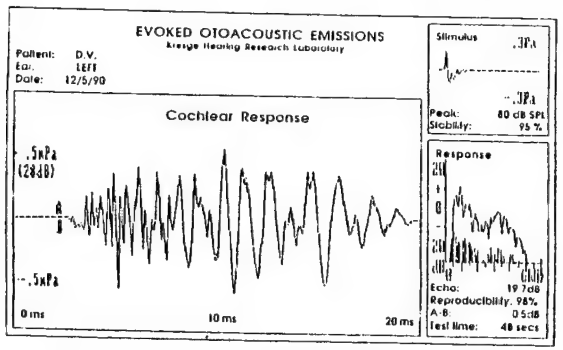
These patients probably have some form of primary auditory neuropathy which makes them poor candidates for hearing aids (Berlin et al., 1993, 1994) . Related evidence suggests that these patients also have other forms of nonauditory primary neuropathy like Charcot-Marie-Tooth syndrome or some similar disease that desynchronizes single unit responses in other motor as well as sensory units (Berlin et al., 1994; Sininger et al., 1995).

SUPPRESSION OF OTOACOUSTIC EMISSIONS

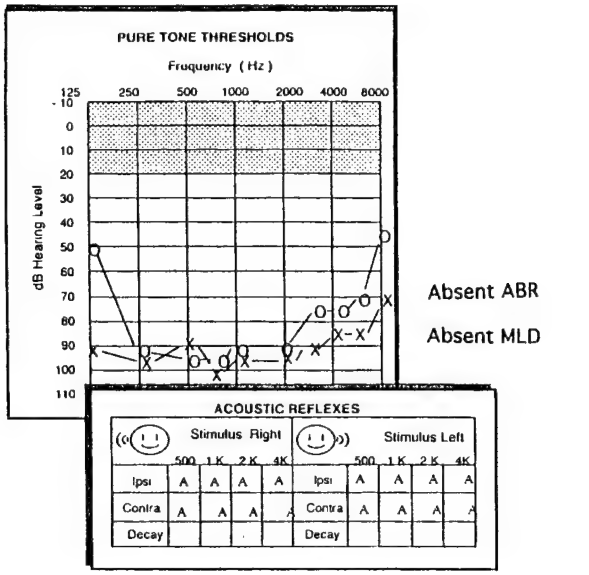
The medial olivocochlear system suppresses segments of outer hair cell activity when activated either contralaterally, ipsilaterally, or bilaterally with an auditory stimulus of sufficient duration (Kujawa et al., 1991; Liberman, 1989; Puel & Rebillard, 1990; Warr & Guinan, 1978; Warr, Guinan, & White, 1986).

In the previous chapter, Hood outlined our techniques for recording otoacoustic emissions and quantifying the efferent suppression that is the hallmark of the integrity of the afferent-efferent loop. In normal hearing subjects the suppression can approach 3 to 4 dB K, the designation we use





Figures 6–2 to 6–4. Audiograms and reflex are typical of those found in some patients with auditory neuropathy. Patients all have normal otoacoustic emissions and absent ABRs, MLDs, middle ear muscle reflexes, and poor speech discrimination in quiet as well as in noise. The patients universally showed no contralateral suppression when assessed by the Wen Kresge Echomaster Program. The kernel point is that the pure tone audiogram alone *cannot* be used to predict hearing aid need, success, or use, unless it is combined at least once with an otoacoustic emissions test showing absent or reduced emissions. *(continued)*



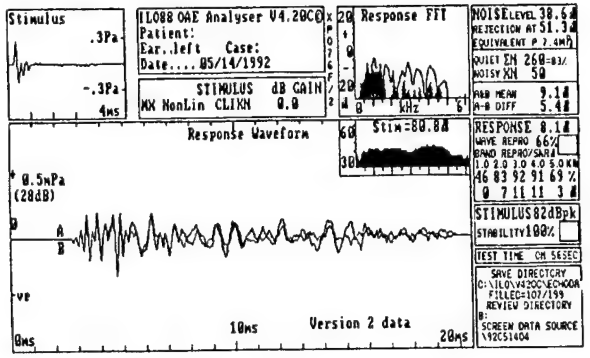


Figure 6–3. See Figure 6–2.

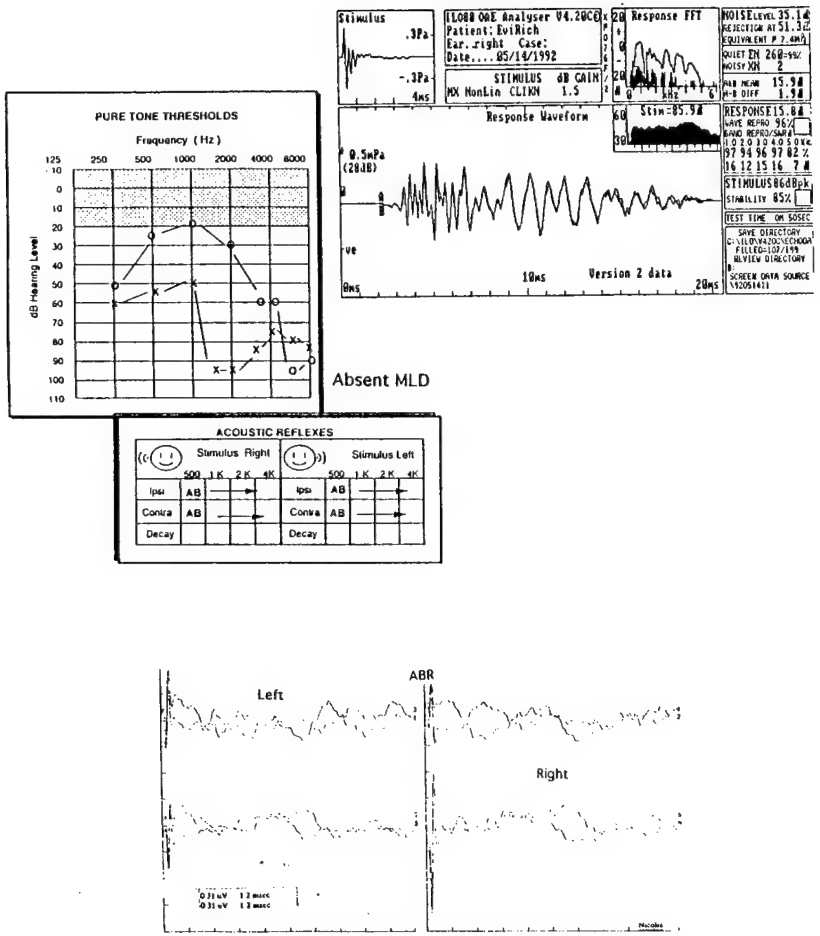
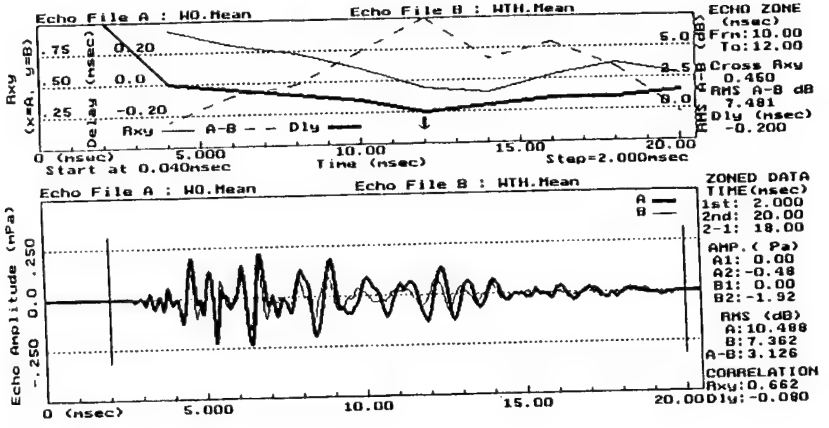
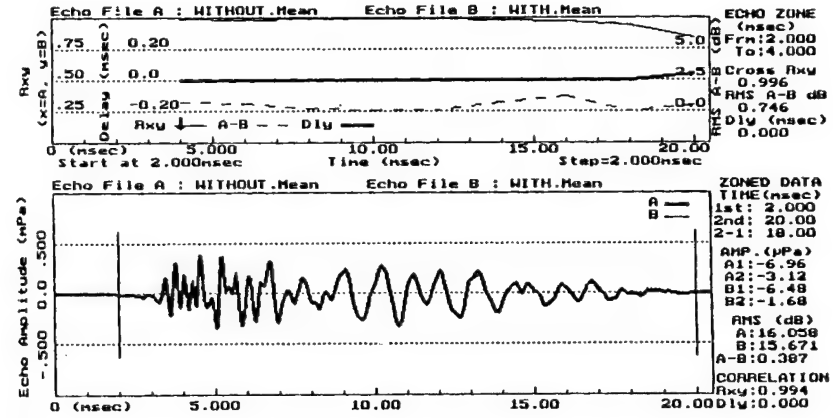


Figure 6–4. See Figure 6–2.



A



B

Figure 6–5. A: A typical click-evoked emission obtained from subjects with normal hearing. **B:** Three virtually parallel lines depicting the absence of suppression in all of the patients with auditory neuropathy.

for the measurements completed with Han Wen's Kresge Echomaster System (1993; Figure 6–5a). Figure 6–5b comes from a patient with auditory neuropathy and shows the pathologic three parallel lines common to all of these patients when studied by efferent suppression techniques. Clearly the nature of their pure tone audiograms is misleading with respect to hearing aid need or success.

Patient 1

This patient was sent to us as part of our search for Ultra-audiometric subjects (Berlin et al., 1978). Her rising audiogram (Figure 6–6) suggested

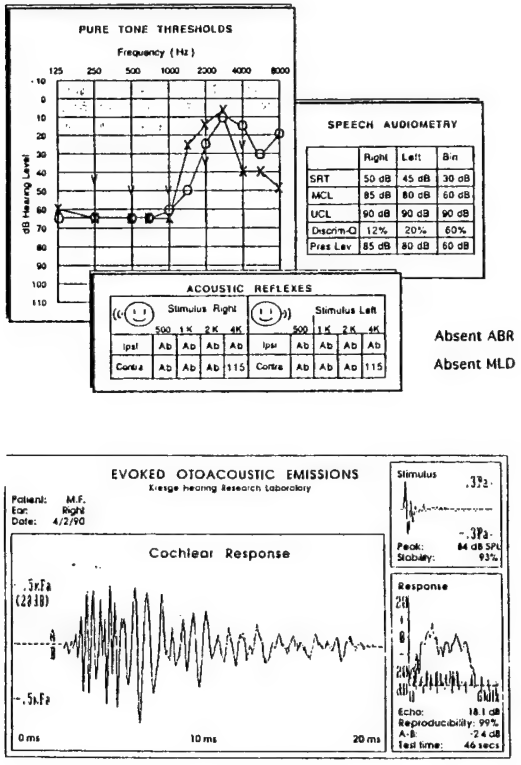


Figure 6–6. Patient with auditory neuropathy and low frequency loss who also has no ABR, no MLD, but normal otoacoustic emissions.

she would be an excellent candidate for hearing aids of one of two types—either a low-frequency emphasis aid with no insertion loss (Killion, Berlin, & Hood, 1984) or one of our body borne translators (Berlin et al., 1978). Her clinical complaint was that she simply could not understand speech in quiet or in noise, and also suffered from mild unsteadiness and poor coordination. She manages quite well in everyday life with the help of her husband, and runs a successful service business. We were not sophisticated enough at the time to recognize the signs that she was not a candidate for ordinary amplification. She tried the K-Bass (Killion et al., 1984) aid with the report that it helped heighten her awareness that someone was talking to her but it did not help her understand speech. We were puzzled at the time over the **absence** of an ABR to clicks or tone bursts around 2 kHz and sent her for a neurological workup. She was diagnosed as having an unspecified leukodystrophy, not MS, and has had no further clarification of her status since then, other than her disease resembles Charcot-Marie-Tooth syndrome but is slightly different (Starr & Picton, personal communication).

Audiological Findings

Her middle ear muscle reflexes were absent despite normal tympanometry. Also absent were any release from masking during MLD testing (Hirsh, 1948) and any synchronous ABR discharge, either to clicks or tone bursts around the zone of her normal hearing. Most surprising, however, were her unusually large and spectrally complex otoacoustic emissions which conventional wisdom predicted would be absent except in a narrow zone between 2 and 3 kHz. (Figure 6–6).

She showed complete absence of contralateral suppression of her otoacoustic emissions at any combination of click intensities and noise levels we used (ranging from 60 to 80 dB peak SP). Because of the absence of both the first wave of the ABR and the total absence of efferent suppression, we conclude that the primary neurons are not synchronous enough to activate an efferent outflow from the brainstem (Berlin et al., 1994).

Patient 2

This 40-year-old woman came to us originally carrying a diagnosis of Charcot-Marie-Tooth syndrome passed down from her father. Her sister is similarly afflicted and, as a matter of fact, is the next patient in this chapter. Figure 6–3 shows her audiogram, and Figure 6–4 shows the somewhat less severe appearing pure tone loss of her sister. It is reasonable to anticipate that Patient 2 would not have much of an ABR, MLD, or middle ear muscle reflex. But what is surprising is that she has normal

and robust otoacoustic emissions and absolutely no contralateral suppression. The evidence for her systemic neuropathy comes from the absence of sensory nerve response in her right arm, and slowed transmission time and reduced motor response of the right median nerve, with normal response of the ulnar nerve. She had no success with the hearing aids we had prescribed (before we observed her normal otoacoustic emissions data).

Patient 3

This 36-year-old woman, who is the sister of the previous patient, also shows no middle ear muscle reflexes, no ABR, no MLD, and large and robust otoacoustic emissions, a paradoxical finding again in view of her pure tone hearing loss (Figure 6–4). She too showed no contralateral suppression and had no success with any hearing aids we tried.

Patient 4

The first three patients all had abnormal audiograms for which one might prescribe hearing aids if one had no information about otoacoustic emissions. In stark contrast, Patient 4 has a nearly normal audiogram but no ABR, no middle ear muscle reflex response, and would have appeared to be deaf, if we had tested him only with ABRs (Figure 6–2). Since he was brought to us at age 12 with a complaint that he simply couldn't understand other people's speech, he was easy to test behaviorally. That's how we found that, despite his absent ABR, he had normal otoacoustic emissions, no efferent suppression, and no MLDs.

What Do the Outer Hair Cells Do?

Outer hair cells probably contribute to some form of mechanical or electrical amplification of low amplitude acoustic inputs or compression of high amplitude inputs. Among the most illuminating observations in this area are the Mossbauer studies (Ruggero, 1992) summarized in Figure 6–7. Here we see that for a 3 dB input signal at 9 kHz the hair cells reflect a 10,000× amplification. In contrast, when the input signal reaches 80 dB, the hair cells impart a little more than 10× amplification. It is clear that outer hair cells play an important part in whatever compression mechanisms operate in the normal ear. The gain functions of the K-Amp and ReSound hearing aids (Figure 6–8) are qualitatively similar to the gain functions Ruggero observed.

The key issue here is that linear amplification doesn't work very well with ordinary outer hair cell hearing losses. They require a nondistorting

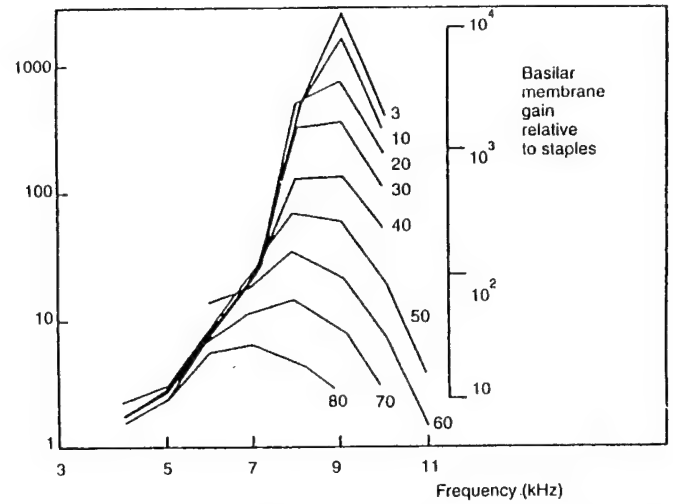


Figure 6–7. Mechanical responses of a chinchilla cochlea to tones. Note that at peak, this gain is more than 10,000 for the lowest stimulus level (3 dB SPL) but drops to values between 10 and 100 for sound levels typical of speech (60 dB SPL to 80 dB SPL). Taken with permission from Ruggero (1992).

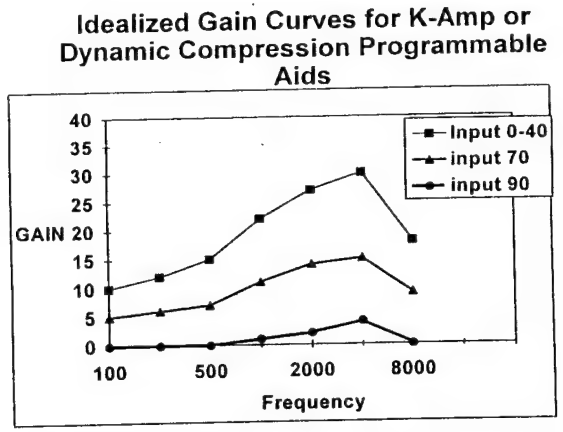


Figure 6–8. An idealized gain curve for K-Amp or dynamic compression programmable aids. Notice how these curves mimic the hair cell reducing gain functions shown in Figure 6–6.

decreasing gain system with increasing input which compensates for the disruptive effects of recruitment on the complex speech signal. It is this expansion of the loudness of vowel peaks and loss of perceived consonant-vowel relationships in speech that will be the topic of the next presentation (Villchur, 1973, 1974).

SUMMARY AND CONCLUSIONS

Otoacoustic emissions and ABR, when used together, form a powerful combination, offering insight into preneural as well as neural function in the cochlea. If emissions are present, patients are *not* hearing aid candidates. Conversely, it is the *absence* of outer hair cell echoes, in the presence of robust and synchronous ABRs at high intensities, that suggests that patients with hearing losses from 20 to 70 dB HL are *good* candidates for dynamic compression aids. These aids compensate for the presumed loss of the low-level amplification ascribed to the outer hair cells, which bring faint sounds into smooth, broad-band, audibility.

Unfortunately, many people are living today with "yesterday's diagnosis": *You have "nerve deafness" and hearing aids will be of limited value.* The fact is that linear aids with restricted frequency responses and ordinary peak clipping simply drove many patients to take the aids off. The aids made vocalic segments of speech disproportionately loud and hence distorted to the listener who was hearing impaired. Because of their restricted frequency response, these aids also limited the quality and number of auditory cues patients could use to hear both in noise and in crowds the way people with normal hearing do.

The kernel message: it is no longer appropriate to fit a linear hearing aid to patients with hair-cell-based losses from 20 to 75dB HL. High fidelity dynamic compression aids that make low-level signals uniformly and smoothly audible are useful answers to the sharp and uneven loudness growth of segments of the speech code for people with recruitment.

Acknowledgments: NIDCD Center Grant P0 1 DC-000379, Training Grants T32-DC-00007, Department of Defense Neuroscience Center Grant via N. Bazan, Kam's Fund for Hearing Research, The Kleberg Foundation, Lions' Eye Foundation and District 8-S Charities. Special support for the entire document and this chapter as well came from Dr. S. Singh, General Hearing Instruments, Etymotic Research, Dr. A. Lippa and Hearing Innovations, and NDIB-BMDR 1549.

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Appendix #16
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Bobbin and Berlin

SEVIER

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HEARING RESEARCH

Contralateral suppression of transient-evoked otoacoustic emissions in humans: intensity effects

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Received 15 September 1995; revised 22 July 1996; accepted 26 July 1996

tract

Transient evoked otoacoustic emissions (TEOAEs) were recorded to clicks presented at peak sound pressures of 50, 55, 60, 65 and dB while continuous contralateral white noise was varied from 10 dB below to 10 dB above the click level. Suppression increased dictably with suppressor noise level for any given click level. However, when the suppressor noise level was held constant, pression was greater for lower click levels. This observation is consistent with the association of suppression of otoacoustic ssions with active cochlear processes and efferent function at low intensity levels.

words: Otoacoustic emission; Transient-evoked otoacoustic emission; Contralateral suppression; Human; Intensity level

Introduction

Studies of the amplitude characteristics of transient-oked otoacoustic emissions (TEOAEs) as a function stimulus intensity agree that TEOAE amplitude in-ases non-linearly with stimulus level and reaches uration at high intensity levels (Wit and Ritsma, 79; Kemp and Chum, 1980; Zwicker, 1983; Zwicker d Schloth, 1984). The effects of intensity level on opression of TEOAEs are less clear and click and ise level combinations yielding the greatest suppres-n have not been systematically studied. For a given mulus level, the amount of suppression increases as e suppressor level increases (e.g. Collet et al., 1990; rlin et al., 1993b). However, when the contralateral ise level is held constant and the stimulus level is ried, the amount of suppression is reported to be atively constant (Veuillet et al., 1991). This finding inconsistent with reports of greater suppression of ditory nerve responses by lower intensity electrical acoustic stimuli with either a saturation or lesser ect at higher intensity levels (e.g. Nieder and Nieder, 1970; Gifford and Guinan, 1987; Warren and Liberman, 1989).

This study had two goals: (1) to determine appropriate click and noise levels for basic and clinical studies of TEOAE suppression, and (2) to address the discrepancy between reported intensity effects on suppression of emissions versus auditory nerve responses. We examined suppression of transient evoked otoacoustic emissions in human subjects while systematically varying both the emission eliciting stimulus and the suppressor noise over a wide range of click and suppressor noise levels.

2. Methods

2.1. Subjects

TEOAEs were obtained from the right ears of 48 subjects (made up of 28 separate individuals) with normal hearing, ranging in age from 12 to 59 years (mean age of 26.6 years; 1 S.D. of 9.3 years). Because of the time involved in testing each subject at multiple noise levels within each click group, the same subjects were not included in all click groups during the 8 month

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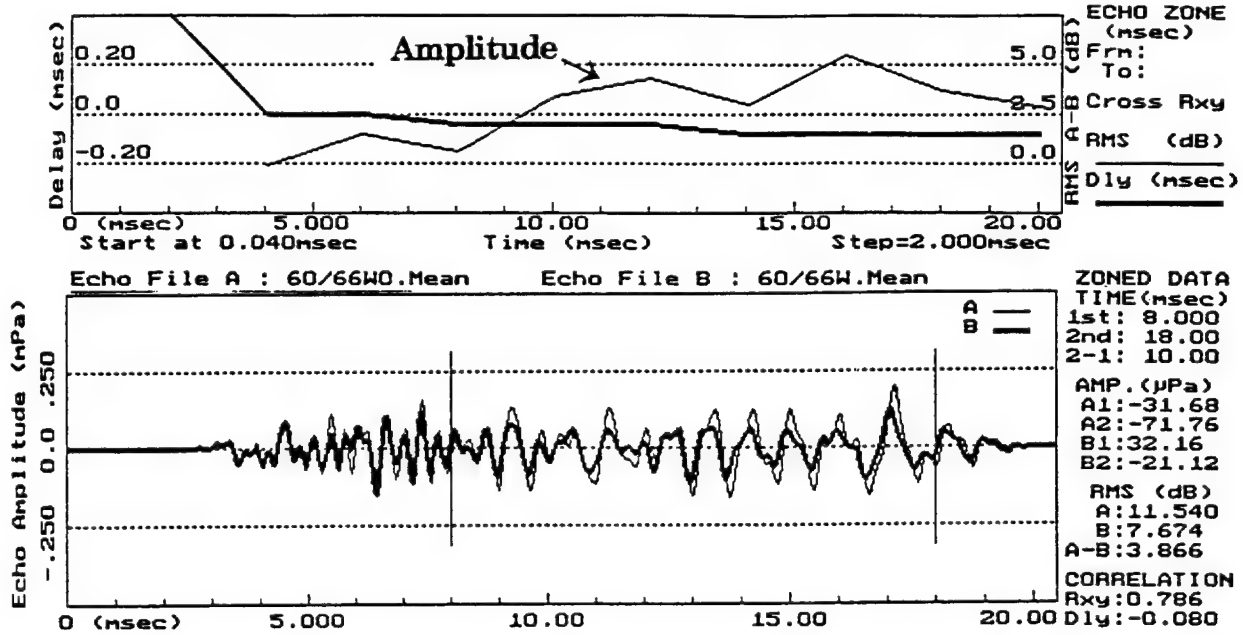


Fig. 1. A sample printout from the Kresge EchoMaster program. In the lower portion of the figure, the time series of emissions obtained without contralateral noise (tracing A, thin line) and with contralateral noise (tracing B, thick line) have been bracketed to focus on the 8–18 m range. The graph in the upper portion of the figure (above the emission tracings) shows the amplitude differences (thin line) across the time series.

duration of the experiment. Eight subjects were evaluated using 50 dB peak SP (sound pressure) clicks (5 females, 3 males), 9 subjects using 55 dB clicks (5 females, 4 males), 9 subjects using 60 dB clicks (6 females, 3 males), 10 subjects using 65 dB clicks (5 females, 5 males), and 12 subjects using 70 dB clicks (6 females, 6 males). The mean age for each of the 5 groups ranged from 25.8 to 28.0 years.

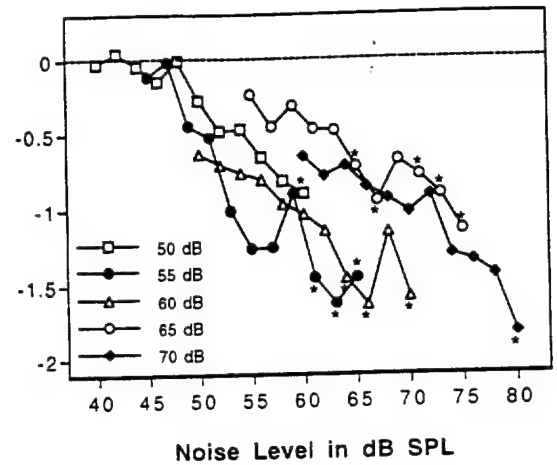
2.2. Methods and apparatus

Eighty microsecond "linear" clicks were presented in an IAC sound-treated room to subjects' right ears at peak sound pressures (peak SP) of 50, 55, 60, 65 and 70 dB and a rate of 50 clicks/s through the standard probe of an Otodynamics ILO88 otoacoustic emission system. The computer processor unit was outside the sound room to maintain low ambient noise in the test environment. "Linear" clicks, defined for the Otodynamics ILO88 system as groups of 4 clicks of the same polarity, were selected rather than the "non-linear" clicks (where the fourth click of a set differs in polarity and intensity) to avoid distortion of emission amplitude. For the 80 μs clicks generated by this system, the average behavioral threshold in a group of normal-hearing subjects was 38 dB peak sound pressure (SP).

Continuous contralateral white noise was generated by a Grason-Stadler 1701 audiometer and presented to the subjects' left ears through an Etymotic Research ER-3A insert earphone. The average behavioral threshold for the noise was 28 dB SPL. The sound pressure level (SPL) of the noise was continuously monitored during testing by an Etymotic Research ER-10 probe microphone placed in the ear canal. The intensity of the suppressor noise was varied from 10 dB below the click peak SP to 10 dB above the click peak SP in 2 dB steps. Thus, when the clicks were at 50 dB peak SP the contralateral white noise varied from 40 to 60 dB overall SPL, when the clicks were at 55 dB peak SP the contralateral white noise varied from 45 to 65 dB overall SPL, etc. Responses to 1040 clicks were averaged across a 20 ms time window for each test condition using artifact rejection. Stimulus levels were monitored for stability and responses were accepted only when the stimulus stability exceeded 80% and the reproducibility of the emissions exceeded 70%. Subjects were presented 3 without-noise and 3 with-noise conditions at each of the 11 noise levels for a total recording of 66 individual TEOAEs at each click level. The without-noise and with-noise conditions were interleaved for each click and noise combination.

2.3. Data analysis

Each of the emissions obtained for each test condition (i.e., without noise and with noise) were viewed separately and compared for response consistency. Then the 3 replications without noise were averaged together and the 3 replications with noise were averaged



2. Mean suppression amplitude for all click and noise intensi-
olotted as a function of the absolute level of the contralateral
ressor noise. Suppression is determined by subtracting the
iout-noise" condition from the "with-noise" condition. Since
ression results in a decrease in the amplitude of the emission,
nore negative the number, the greater the suppression effect.
ie noise levels producing significantly greater suppression than
r noise levels for each click intensity are marked with an aster-
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ether, yielding averages of 3120 sweeps for each test
dition for each subject. Suppression was determined
subtracting the without-noise condition from the
n-noise condition. Thus, greater suppression was
nonstrated with more negative-going difference
es. Rms amplitude differences between without-
se and with-noise conditions were compared using
Kresge EchoMaster (KEM) emissions analysis pro-
m version 3.0 (Wen et al., 1993). A sample printout
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hout-noise and with-noise conditions across the
ie series in 2 ms steps. The amplitude differences,
npared in the present study, are shown by the thin
e in the upper portion of the figure (see arrow).
Amplitude values across the 8–18 ms time period
re averaged to obtain a single number representing
amplitude of each emission. This time period was
ected based upon previous studies showing greater
pression amplitude in later time periods (e.g. Veuil-
et al., 1991; Berlin et al., 1993b). Data in the 18–20
time period were not included since emission ampli-
le was near or below the noise floor. Repeated meas-
es analyses of variance were performed to examine
e effects of the click and suppressor noise intensities.
st-hoc testing used Fisher's PLSD range test. To compare suppression at a common noise level across the 5 click intensity levels, suppression data with contralateral noise levels from 59 to 61 dB SPL were compared for all 5 click levels.

3. Results

3.1. TEOAE amplitude

The mean TEOAE amplitude between 8 and 18 ms without contralateral noise was 4.07 dB SPL for 50 dB peak SP clicks, 5.13 dB SPL for 55 dB peak SP clicks, 9.05 dB SPL for 60 dB peak SP clicks, 5.76 dB SPL for 65 dB peak SP clicks and 7.99 dB SPL for 70 dB peak SP clicks. The use of different subjects at the various click levels is the most likely explanation for the discontinuity in growth of emission amplitude observed at 65 dB.

Since the ILO88 system is most widely used for transient evoked otoacoustic emission testing, we compared the full 20 ms time window amplitude value to the 8–18 ms time window amplitude we use in quantifying suppression. For the 48 subjects in this study, calculation of suppression between 8 and 18 ms was greater for 39 subjects than was suppression calculated across the entire time window which supports previous reports that greater suppression is observed in later time periods (Veuillet et al., 1991; Berlin et al., 1993a). In addition, we observed that some subjects who showed very little suppression across either the full time window or in the averaged 8–18 ms range demonstrated substantial suppression in more restricted time periods between 8 and 18 ms.

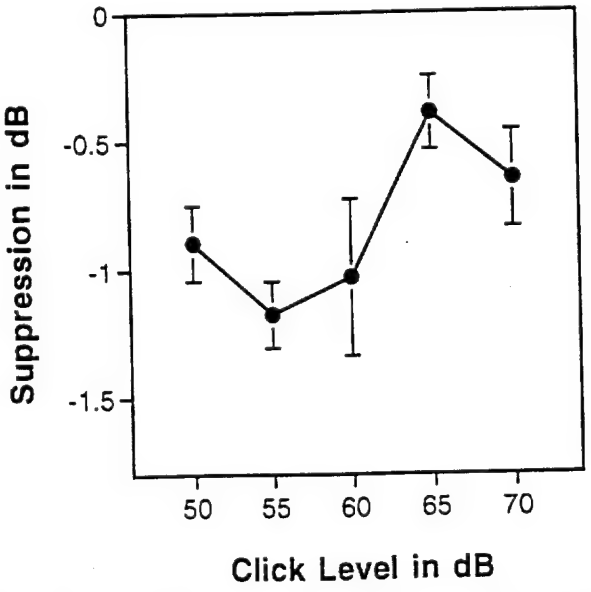

Fig. 3. Mean suppression and 1 S.E. comparing the 5 click intensity levels for contralateral suppressor noise levels of 59–61 dB SPL.

3.2. Comparison of all click and suppressor noise levels

For any given click intensity level, suppression of TEOAEs increased with increasing level of the suppressor noise. Suppression of emissions by higher noise levels was significantly greater ($P < 0.001$) than by lower noise levels for each of the 5 click intensity level groups. Fig. 2 shows the mean suppression for each of the 5 click levels and the 11 suppressor noise levels. For the 50 and 55 dB peak SP clicks, noise levels below 50 dB SPL yielded little or no suppression. Suppression variability across subjects, based upon the standard error of the mean for each of the click and noise intensity combinations, ranged from 0.07 to 0.36 dB with a mean of 0.21 dB.

At each of the 5 click levels, suppression increased systematically as the level of the noise increased from a mean suppression of 0.33 dB when the noise was 10 dB below the click to a mean suppression of 1.38 dB when the noise was 10 dB above the level of the click.

3.3. Comparison of suppression at 5 click intensities and a constant noise level

For a constant 60 dB SPL noise level (or the average of 59 and 61 dB SPL), suppression was greater for click intensities of 60 dB and below. The mean suppression and 1 S.E.M. for the 5 click levels are plotted in Fig. 3 as a function of a 60 dB SPL noise level for clicks at 50, 60 and 70 dB peak SP and an average of the 59 and 61 dB SPL noises for clicks at 55 and 65 dB peak SP. Greater suppression was observed for 50, 55 and 60 dB peak SP clicks than for 65 and 70 dB peak SP clicks. Post-hoc tests showed that suppression for 50 and 55 dB peak SP clicks was significantly greater (predefined at $P < 0.05$) than suppression for 65 dB peak SP clicks.

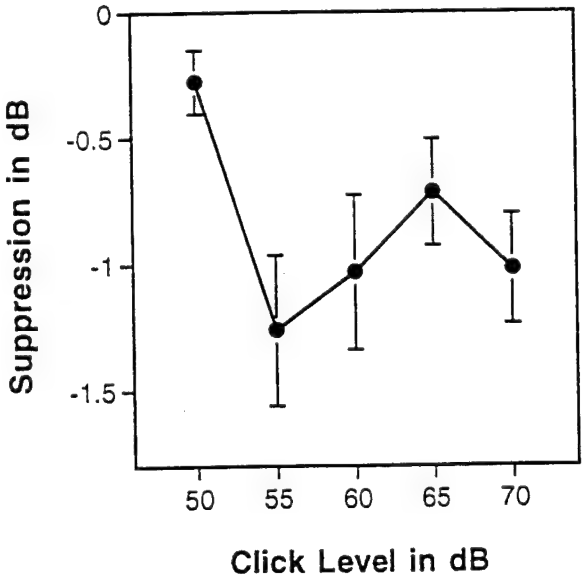

Fig. 4. Mean suppression and 1 S.E. for click and noise levels where the noise level increased along with the click level.

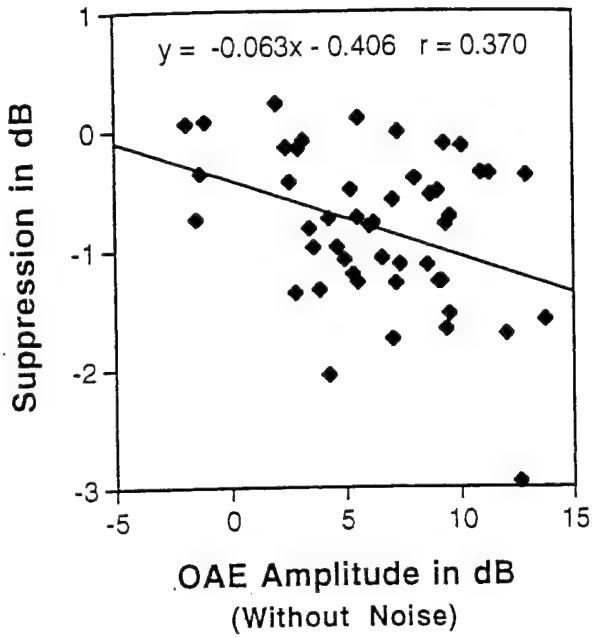

Fig. 5. Scatterplot of emission amplitude in the without-noise condition plotted against the amount of amplitude suppression with 59–61 dB SPL noise levels for each subject.

3.4. Comparison of suppression at equal click and noise levels

It was reasonable to expect that weak noises would have little effect on stronger clicks (see Fig. 3). Therefore, to evaluate suppression when the click and noise levels were similar, we studied the data when 50 dB SP clicks were paired with 50 dB SPL noise, 55 dB SP clicks with 55 dB SPL noise, etc. The least suppression was observed for 50 dB clicks and this was significantly ($P < 0.05$) less suppression than obtained with 55, 60 or 70 dB clicks. The mean suppression and 1 S.E. are shown in Fig. 4.

3.5. The relationship of suppression to TEOAE amplitude

Since different subjects have different amplitude emissions and since different subjects comprised the different click level groups, we wished to determine whether emission amplitude and suppression amplitude were related. Emission amplitude in the without-noise conditions was plotted against suppression amplitude for each subject for both the data acquired with the suppressor noise at or near 60 dB SPL and for all click and suppressor noise levels. The scatterplot in Fig. 5

licates a correlation of 0.37 between TEOAE ampli-
le and suppression amplitude for the 59–61 dB SPL
ise data. A similar correlation ($r = 0.351$) was ob-
ned when both the click and suppressor noise inten-
es varied.

Discussion

This study showed that changes in suppression of
EOAEs as a function of click and contralateral sup-
essor noise intensity levels were generally monotonic
r increasing noise at any given click level but non-
onotonic across click levels. Greater suppression was
served for lower intensity level clicks when the sup-
essor noise level was held constant. The amount of
ppression did not appear related to TEOAE ampli-
de.

1. Effects of click intensity on suppression

Greater amplitude suppression for emissions was ob-
ined with lower intensity level clicks when the inten-
y of the contralateral noise was at or near 60 dB SPL.
his finding is inconsistent with the data of Veuillet et
. (1991) who found no decrease in suppression for
gher intensity clicks. They presented clicks at levels
om 48 to 75 dB peak SP and, in contrast with the
esent study, held the contralateral noise level constant
50 dB SPL. Since we did not use a noise level of 50
B SPL for all click levels, we cannot directly compare
e results of the two studies.

Decreases in suppression at higher intensity levels are
nsistent with suppression effects observed for N_1 with
oth electrical and acoustic stimulation in studies with
ts. Gifford and Guinan (1987) reported that electrical
imulation of efferent fibers at the floor of the fourth
ntricle depressed N_1 to a greater extent at low and
oderate click levels than at high click levels. Warren
d Liberman (1989) showed that the extent of suppres-
on of auditory nerve responses is related to the level
f the response-eliciting stimulus. They found that the
fects of contralateral sound on rate-level functions
ntained a region of dynamic growth where discharge
te increased rapidly with level and a saturation region
here rate is maximal with little or no change with
vel. A similar finding was reported by Nieder and
Nieder (1970) who showed that electrical stimulation
educed the magnitude of the action potential for low
ut not high intensity acoustic stimuli.

What needs to be reconciled is the difference in the
nctions obtained when the noise is held constant
hile the click level increases (Fig. 3) versus the func-
on when the noise and click levels both increase in
ndem (Fig. 4). It is reasonable to expect that a 60
B noise would be more effective in suppressing emis-
sions from weaker than stronger clicks. When the click and noise levels increase in tandem, we see a decrease at 65 dB with an increase in suppression at 70 dB which requires some explanation or at least speculation. We believe that two possibilities are worthy of future study: (1) the source of the suppression shifted from an active to passive mechanism; (2) there was participation of the middle ear muscle reflex as noise levels exceeded 70 dB SPL. As frank speculations, they will require additional experimentation to afford any credibility.

4.2. Effects of suppressor noise level

When the intensity level of the contralateral suppressor noise was successively increased, suppression amplitude generally increased for each of the click levels. This observation is supported by other studies that have demonstrated the effects of noise level on contralateral suppression (Collet et al., 1990; Ryan et al., 1991; Veuillet et al., 1991). Collet et al. (1990) and Veuillet et al. (1991) presented linear clicks at average intensities of 60–63 dB peak SP and compared emissions to those obtained with contralateral broad-band noise ranging from 0 to 50 dB SPL. Noise levels from 30 to 50 dB SPL significantly reduced the amplitude of the emissions and no significant changes were observed for noise levels below 30 dB SPL. Ryan et al. (1991) reported suppression of clicks with broad-band noise at 50 dB SL (sensation level, or dB above threshold) and some subjects where 20 dB SL noise produced suppression. In our experiment, 20 dB SL would have been about 48 dB SPL.

4.3. TEOAE amplitude and its relation to suppression amplitude

Increases in stimulus level within subjects result in monotonic increases in emission amplitude with saturation at higher intensities (Wit and Ritsma, 1979; Kemp and Chum, 1980; Zwicker, 1983; Zwicker and Schloth, 1984). We attribute part of the non-monotonic increases in the "without-noise" clicks to the use of different subjects at each of the click levels. Although all subjects had normal hearing thresholds and no middle ear problems, there is considerable variability among subjects in emission amplitude, attributable to a number of factors, including age and gender. Males and females were included in each group and the mean age of each group was similar. Analysis of age relative to emission amplitude did not show any consistent trends. While a slight trend toward more suppression with higher emission amplitude was noted, examination of the scatterplots and the correlations suggest difficulty in predicting one value from the other. These data suggest that the absolute amplitude of the emissions is not strongly related to suppression amplitude (see Fig. 5).

4.4. Possible influence of the middle ear muscle reflex and acoustic crossover

Greater suppression with lower intensity level stimuli decreases the likelihood that the middle ear muscle reflex is the primary explanation for the suppression observed in this experiment. While the status of the middle ear system clearly affects otoacoustic emission amplitude, several investigators have argued against a major role of the middle ear muscle reflex in suppression and have demonstrated suppression in patients with severed stapedius muscles and in patients with impaired function of the facial nerve (Collet et al., 1990; Veuillet et al., 1991; Berlin et al., 1993b).

Acoustic crossover of the contralateral suppressor noise is also unlikely since the insert earphones used in this study have interaural attenuation of 60–90 dB (Killion et al., 1985), with greater attenuation in the lower frequencies. The minimal contribution of crossover is consistent with transcranial attenuation measurements of 75 dB reported by Ryan et al. (1991) and the lack of interference by 80 dB SPL noise reported by Collet et al. (1990).

4.5. Clinical measurement of contralateral suppression of TEOAEs

Based on the observations in this study of maximal suppression for 55, 60 and 70 dB clicks with varying noise levels and maximal suppression for 55 and 60 dB clicks with a constant noise level, we now measure suppression of transient evoked otoacoustic emissions using linear clicks at 55 or 60 dB peak SP with the overall intensity level of the noise set 5 dB higher than the click. We avoid 70 dB clicks to minimize the risk of major participation of the middle ear muscle reflexes. We use 60 dB peak SP clicks and 65 dB SPL white noise when control emissions obtained at 55 dB peak SP are less than 2 or 3 dB SPL in overall amplitude. With these test parameters, we believe that we are able to maximize study of suppression of TEOAEs by contralateral noise while minimizing contamination by acoustic crossover and the middle ear muscle reflex.

Acknowledgments

This work was supported by NIH NIDCD P01-DC00379, NIH NIDCD T32-00007, Kam's Fund for Hearing Research, the Kleberg Foundation, and the Louisiana Lions Eye Foundation. Portions of this study were reported at the 1994 Midwinter Research Meeting of the Association for Research in Otolaryngology.

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126 Chronic low-level noise exposure alters distortion product otoacoustic emissions

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Chen *et al.* (*Hear. Res.* in press, 1995) recently reported an altered response to the application of ATP in outer hair cells (OHCs) isolated from guinea pigs continuously exposed for 10 or 11 days to a 65 dB SPL (A-scale) narrowband noise (1.1 - 2 kHz). The primary goal of the present study was to test the hypothesis that the continuous low-level noise used by Chen *et al.* alters cochlear function. Cubic ($2f_1$-f_2) and quadratic (f_2-f_1) DPOAEs, as well as, the amount of contralateral suppression of DPOAE amplitudes were chosen for study. Responses were recorded in urethane-anesthetized guinea pigs with sectioned middle ear muscles. The animals had either been exposed to the low-level noise for 3 or 11 days or not exposed at all (n = 13 animals per group). Results demonstrate that this noise induces frequency-dependent and very localized reductions in $2f_1$-f_2 DPOAE input-output functions. However, the f_2-f_1 DPOAE input-output functions and the time-varying amplitude characteristics of f_2-f_1 DPOAE appear to be insensitive to the noise exposure. No noise-related changes were found in the amount of contralateral suppression between the different exposure groups, with the exception of one unexplainable data point (f_2-f_1 DPOAE = 0.5 kHz; day 3) where it was reduced. The $2f_1$-f_2 DPOAE amplitude alterations lend support to the conclusions of Chen *et al.* that chronic low-level noise exposure induces molecular changes in the OHCs which may, in turn, alter cochlear function.

DAMD 17-93-V-3013, AASERT DAAH04-94-G-0111, NIH P01-DC00379, R01-DC00722, T32-DC00007, Kam's Fund for Hear. Res., & LA. Lions Eye Fnd.

136 "Conditioning" the auditory system with continuous *vs.* interrupted noise of equal acoustic energy: Is either exposure more protective?

**R.A. Skellett, M. Fallon, R.P. Bobbin (Kresge Hearing Research Lab., LSUMC, New Orleans)*

The temporal pattern of noise exposure is an important factor which affects both the magnitude of cochlear damage and the pattern of auditory sensitivity change over time. Continuous noise exposure is more damaging to the cochlea than an interrupted exposure of equal energy (Bohne *et al., Ann Otol. Rhinol. Laryngol.* 94: 122, 1985; Fredelius and Wersäll, *Hear. Res.* 62: 194, 1992). Continuous noise exposure results in a pattern of sensitivity change referred to as an asymptotic threshold shift (ATS; Carder and Miller, J. *Speech Hear. Res.* 15: 603, 1972). Interrupted noise exposure results in a progressive resistance to threshold shift known as "toughening" (Clark *et al., J. Acoust. Soc. Am.* 82: 1253, 1987; Subramaniam *et al., Hear. Res.* 52: 181, 1991). Both exposure schedules have been used in "conditioning" experiments and both have been effective in providing protection against subsequent traumatizing noise (Canlon *et al., Hear. Res.* 34: 197, 1988; Campo *et al., Hear Res.* 55: 195, 1991). The purpose of this investigation is to determine if differences exist in the amount of protection afforded by prior "conditioning" of the auditory system with continuous or interrupted noise of equal acoustic energy.

Guinea pigs were "conditioned" with an octave band of noise (1-2 kHz) for 24 hours per day for 11 days at 89 dB SPL (n=14) or for 6 hours per day for 11 days at 95 dB SPL (n=14). After a one week rest period, both groups were continuously exposed for 3 days to the same noise spectrum at 105 dB SPL. A third group of animals (n=14) was only exposed to the high-level exposure. All animals were given 4 weeks to recover from the high-level exposure. Cubic ($2f_1$-f_2) DPOAEs were then recorded in urethane-anesthetized guinea pigs with sectioned middle ear muscles.

Preliminary results suggest that an interrupted "conditioning" noise may provide more protection against subsequent damaging exposures than a continuous exposure of equal acoustic energy. However, the results obtained thus far are not conclusive and require further experimentation to determine if statistically significant differences exist between the two exposure groups.

DAMD 17-93-V-3013, AASERT DAAH04-94-G-0111, NIH P01-DC00379, R01-DC00722, T32-DC00007, Kam's Fund for Hear. Res., & LA. Lions Eye Fnd.

526 ATP does not generate a current response in outer hair cells of rat cochlea

C. Chen, C.S. LeBlanc, R.P. Bobbin (Kresge Hearing Research Lab., LSUMC, New Orleans)

Increasing evidence suggests that ATP may act as a neurotransmitter and/or modulator in the mammalian cochlea (Kujawa *et al. Hear. Res.* 78:181, 1994). Extracellular application of ATP, related agonists, and ATP antagonists significantly affect cochlear potentials in guinea pig. In isolated outer hair cells (OHCs) of guinea pig, ATP depolarizes the cell membrane by inducing non-selective cation currents. However, nothing is known about the effect of ATP on rat OHCs. The purpose of the present study was to compare the ATP response in OHCs from guinea pig to the ATP response in OHCs from rat. The whole-cell configuration of the patch-clamp technique was used. OHCs were isolated from pigmented guinea pigs and Sprague Dawley rats. Extracellular application of 100 μM ATP or ATP-γ-S induced a large inward current in OHCs of guinea pig recorded at -60 and -100 mV. The same concentration of ATP or ATP-γ-S did not induce any detectable response in rat OHCs at -60 mV or at -100 mV. However in these same rat OHCs, 100 μM ACh induced an inward current at -100 mV and an outward current at 0 mV. When K^+ was substituted by N-methyl-glucamine (NMG^+) in the pipette solution, no current was induced in rat OHCs by ATP. Adenosine, AMP and ADP did not produce a response in rat or guinea pig OHCs. In both rat and guinea pig, ATP produced a very large inward current in supporting cells closely associated with the OHCs. Our results suggest that there may be no or very few ATP receptors in OHCs of rat cochlea. This is in contrast to guinea pig cochlea where ATP receptors on OHCs appear to be large in number. On the other hand, supporting cells in both rat and guinea pig seem to have a similar number and type of ATP receptors.

Supported by NIH grant R01-DC00722, DAMD 17-93-V-3013, Kam's fund for Hearing Research, and the Louisiana Lions Eye Foundation

771 Differences in the degree of protection provided by interrupted vs. continuous sound conditioning

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Prior exposure to moderate-level acoustic stimulation (conditioning) can reduce and/or prevent the deleterious effects of subsequent higher level exposures (*Canlon et al., 1988; Campo et al., 1991*). Both continuous and interrupted schedules of moderate-level noise have been used as conditioning exposures, and both schedules have been effective in providing protection against subsequent noise trauma. The purpose of this study is to test the hypothesis that differences exist in the amount of protection provided by prior sound conditioning with continuous vs. interrupted, moderate-level noise of equal acoustic energy.

Guinea pigs were conditioned with an octave band of noise (1-2 kHz) for 24 hours per day for 11 days at 89 dB SPL or for 6 hours per day for 11 days at 95 dB SPL. After a one week rest period, both groups of "conditioned" animals were continuously exposed for three days to a 105 dB SPL noise with the same spectral characteristics. Another group of animals was exposed only to the 105 dB SPL noise. All animals exposed to the high-level noise were given 4 weeks to recover. Differences in the amount of protection were determined by monitoring the changes in cubic ($2f_1$-f_2) distortion product otoacoustic emission (DPOAE) amplitude growth functions.

Results suggest that there are significant differences in the degree of protection provided by prior sound conditioning with the continuous and interrupted schedules of moderate-level noise used in this study. Specifically, the interrupted conditioning protocol appears to afford some degree of protection against the damaging effects of the traumatizing noise exposure. However, the frequency region protected is limited to frequencies above the noise exposure band. Conversely, there is a lack of any consistent and sizable protective effect found across the entire test frequency range for the continuous conditioning protocol. Therefore, while neither conditioning protocol was effective in providing protection in the low frequency range (frequencies encompassing the noise exposure band and extending down to 1/2 octave below the lower cutoff frequency), the interrupted sound conditioning protocol was more effective than the continuous conditioning protocol in the frequency region above that of the noise exposure band.

DAMD 17-93-V3013, AASERT DAAH04-94G0111, NIH P01-DC00379 R01-DC00722, T32-DC00007), Kam's Fund Hear. Res., LA Lions Eye Found.

54 The ATP antagonist, suramin, prevents the time related decrease of the quadratic (f_2-f_1) otoacoustic distortion product

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ATP suppressed the cubic distortion product otoacoustic emission (DPOAE) when applied into the perilymph (*Kujawa, et al., Hear. Res. 76, 87-100, 1994*). The ATP antagonist, cibacron blue, but not suramin, suppressed the cubic DPOAE (*Kujawa et al., Hear. Res. 78, 181-188, 1994*). Therefore, we studied the effects of cibacron blue and suramin on single OHCs and Deiters' cells to elucidate the mechanisms of action in suppressing DPOAEs. The actions of suramin on quadratic DPOAEs were also explored. Drugs were applied to the perilymph compartment of the guinea pig cochlea in artificial perilymph at 2.5 microliter/ min. The cubic ($2f_1$-f_2 = 5 kHz) and quadratic (f_2-f_1 = 1.25 kHz) DPOAEs were elicited by equilevel primaries (f_1 = 6.25 kHz; f_2 = 7.5 kHz). Changes in the amplitude of the quadratic DPOAE induced by continuous sound stimulation with the primaries (L1 = L2 = 60 dB SPL) were recorded over time (*Kujawa et al., Hear. Res. 97, 153-164, 1996*). Both ligand and voltage induced currents were monitored using the whole-cell configuration of the patch-clamp technique (*Chen et al., Hear. Res. 86, 25-33, 1995*).

Cibacron blue (100 micromolar), but not suramin (100 micromolar), was found to reduce the outward K^+ current in Deiters' cells and increase the K^+ current in OHCs. Both antagonists (100 micromolar) suppressed the current induced by 5-10 micromolar ATP in Deiters' cells and OHCs. The time related decrease of the quadratic DPOAE induced by continuous sound stimulation with 60 dB SPL primaries was reversibly prevented by suramin (100 micromolar). Suramin also increased the subsequent input/output function of the quadratic DPOAE. Results suggest that endogenous ATP acting on receptors on Deiters' cells and/or OHCs may contribute to the time related decrease of the quadratic DPOAE induced by continuous sound stimulation. Also, some of the effects of cibacron blue on the cubic DPOAE reported earlier may have been due to the effect of cibacron blue on K^+ currents.

Supported by NIH (P01-DC00379, R01-DC00722), DAMD 17-93-V-3013, Kam's Fund for Hearing Research, and Louisiana Lions Eye Foundation

294 Adenosine triphosphate (ATP) induces outer hair cell death

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Adenosine Triphosphate (ATP) concentration in storage granules within cells has been reported to be greater than 1 M (*Gordon, J.L., Biochem J. 233, 309-319, 1986*). Activation of ATP receptors on outer hair cells (OHC) leads to a rapid volume loading, and potential death (*Housley et al., Active Hearing, ed. Flock, A., Ottoson, D. and Ulfendahr, M., Pp. 221-238, 1995*). It is hypothesized that within the cochlea, an influx of ATP into the extracellular space of the organ of Corti may be the cause of noise-induced OHC death. The purpose of this study is to ascertain whether ATP causes OHC death *in vivo* and *in vitro*.

In vivo, ATP (60 mM) or sodium gluconate (60 mM) was applied to the perilymph compartment of the guinea pig cochlea in artificial perilymph at 2.5 µl/min for 2 hrs and the animals allowed to recover from the surgery. Cubic ($2f_1$-f_2) distortion product otoacoustic emissions (DPOAE) and cochlear potentials (cochlear microphonics, compound action potential) were monitored 2-3 weeks after drug delivery. *In vitro*, isolated OHCs were placed into a 100 µl drop of external medium and continuously perfused with additional medium at a rate of 100 µl/min. Drugs were applied isosmotically.

In vivo, 2-3 weeks later, cochlear potentials and DPOAEs could be recorded from sodium gluconate treated cochlea, but not ATP treated cochlea. *In vitro*, sodium gluconate (30mM) did not significantly affect OHCs, whereas, ATP (30 mM) caused OHCs to either burst or leak out their cellular contents thus, becoming visually "ghostly." The bursting was only at the apical end of the cell, just below the cuticular plate. This is in contrast to cell bursting at the basal end caused by hypotonic solutions. At 20 mM, ATP caused cell death in short (<70 µm) OHCs, but not in long (>70 µm) OHCs At 10 mM, 1 mM, and 100 µM concentrations, ATP only caused cell shortening, but not cell death.

Results demonstrate that at relatively high concentrations, ATP causes cell death. These concentrations may be present in the extracellular space during intense noise exposure. Future studies may be able to utilize ATP antagonists to prevent noise induced hearing loss.

*Supported by NIH grant PO1-DC00379, DAMD 17-93-V-3013,
Kam's Fund for Hearing Research, and the Louisiana Lions Eye Foundation*